EFFECT OF CHROMIUM TOXICITY ON DENGUE VIRUS INFECTION IN MICE

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CERTIFICATE OF THE SUPERVISOR

*This is to certify that the work entitled "**EFFECT OF CHROMIUM TOXICITY ON DENGUE VIRUS INFECTION IN MICE**" is a piece of research done by Miss Richa Shrivastava under our guidance and supervision for the degree of Doctor of Philosophy in Chemistry of Bundelkhand University, Jhansi (U.P.), India. That the candidate has put in an attendance of more than 200 days with me.*

To the best of our knowledge and belief the thesis:

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iii *fulfills the requirement of the ordinance relating to the Ph.D degree of the University; and*

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Dedicated to My Parents

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LIST OF ABBREVIATIONS

µCi	Micro Curie
µg	Micro gram
µl	Microleter
[3H]	Tritium
CF	Cytotoxic Factor
Co A	Co-enzyme A
Con A	Concanavalin A
CPM	Counts per minute
Cr(III)	Trivalent Chromium
Cr(VI)	Hexavalent Chromium
DF	Dengue Fever
DHF	Dengue Haemorrhagic Fever
DLC	Differential leucocyte count
DMSO	Dimethyl sulphoxide
DV	Dengue virus
EDTA	Ethylene diamine tetra acetic acid Sod. salt
FCS	Fetal calf serum
GIT	Gastrointestinal Tract
gm	Gram
Hb	Haemoglobin
hCF	Human Cytotoxic Factor
Hct	Haematocrit
ic	Intracerebral
$K_2Cr_2O_7$	Potassium dichromate
KCl	Potassium Chloride
KH_2PO_4	Potassium Dihydrogen Ortho phosphate
LD_{50}	Half of the Lethal Dose
Mϕ	Macrophages
mCF	Murine cytotoxic Factor
MCH	Mean corpuscular haemoglobin

MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MEM	Minimal essential media
mg	Milli gram
MIC	Minimal inhibitory concentration
mM	Milli molar
MPV	Mean platelet volume
MTT	[3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
nm	Nanometer
OD	Optical density
PBS	Phosphate buffered saline
PDW	Platelet distribution width
pH	Potential of hydrogen
PHA	Phytohaematoglutinin
Plt	Platelet count
PPM	Parts per million
RBC	Red blood cell
ROS	Reactive oxygen species
rpm	Rounds per minute
SD	Standard deviation
TLC	Total leucocyte count

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Introduction

INTRODUCTION

Chromium is one of the naturally occurring heavy metal found in environment in many forms, the commonest stable oxidation states being trivalent (Cr III) and hexavalent (Cr VI). Cr(VI) compounds have been declared as a potent occupational carcinogen by IARC (1990) through epidemiological studies among workers in chrome plating, stainless-steel, and pigment industries. The major non-occupational source of chromium for humans is food such as vegetables, meat *etc.* (Hertel, 1986). Other potential non-occupational sources include urban air, hip or knee prostheses and cigarettes (U.S. EPA 1984). The reduction of Cr(VI) to Cr(III) results in the formation of reactive intermediates that contribute to the cytotoxicity, genotoxicity, and carcinogenicity of Cr(VI)-containing compounds.

Chromium enters the body through the lungs, gastro-intestinal tract, and to a lower extent through skin (Hamilton and Wetterhahn, 1988). Inhalation is the most important route for occupational exposure, whereas non-occupational exposure occurs via ingestion of chromium containing food and water (Langard, 1982; Hertel, 1986). Most of the chromium absorbed by the body is distributed in the lungs, liver, kidneys, RBC, plasma, spleen and bone marrow (Langard, 1982). Cr (VI) enters into the cells through membrane anionic transporters while Cr (III) does not. Intracellular Cr (VI) is metabolically reduced to the ultimate Cr (III). It is therefore in the interest of the body to reduce the toxic Cr (VI) to less toxic form, the Cr (III).

Dengue is a mosquito-borne virus infection which is found in tropical and sub-tropical regions around the world, predominantly in urban and semi-urban and now in rural areas also. The virus produces a benign self-limiting illness, the dengue fever (DF) or a life-threatening serious illness, the dengue haemorrhagic fever (DHF). The prevalence of dengue has grown dramatically in recent decades. The disease is now endemic in more than 100 countries.

Some 2500 million people – two fifths of the world's population – are now at risk from dengue. WHO currently estimates there may be 50 million cases of dengue infection worldwide every year. An estimated 500 000 cases of DHF require hospitalization each year, of whom a very large proportion are children (Halstead, 2002; Agarwal *et al.*, 1999). Dengue is endemic in India with frequent epidemics of DF and DHF.

Viral virulence and immune responses have been considered as two major factors responsible for the pathogenesis of DHF. The immunopathological mechanisms appear to include a complex series of immune responses. A rapid increase in the levels of cytokines and chemical mediators apparently plays a key role in inducing plasma leakage, shock and haemorrhagic manifestations (Chaturvedi *et al.*, 1999a; 2000). Dengue continues to be a Global challenge because the pathogenesis of DHF is not fully understood.

The cells of the immune system form a strong line of defence against foreign substances. Following entry into body Cr (VI) reaches the cells of the immune system which try to eliminate it and in the process their functions may also be adversely affected.

Cr (VI) is highly toxic to all forms of living organisms and is mutagenic in bacteria (Losi *et al.*, 1994). The presence of chromate in the environment inhibits most microorganisms but also promotes the selection of resistant bacteria. The processes by which the microorganisms interact with the toxic metals enabling their removal/and recovery are biosorption, bioaccumulation and enzymatic reduction. Microorganisms have evolved resistance mechanism to select resistant variants to deal with metal toxicity as the result of exposure to metal contaminated environments. The commonest route of entry of chromium is through drinking water and food. Intestines have a huge population of bacteria and the caecum habours the largest number (Siman and Gorbach, 1986). Further, some of the bacteria bioaccumulate large quantity of

Cr and bring down the residual concentration of Cr (VI) in 24 h (Srinath *et al.*, 2002). Thus, bacteria may play an important role in protecting body from the toxicity of ingested chromium. The resident bacterial flora of the gastro-intestinal tract is exposed to Cr through ingestion but the literature on the effect of Cr on resident gut microflora is scarce (Francisco *et al.*, 2002; Viti *et al.*, 2003). It was, therefore, considered important to investigate the effect of chronic ingestion of chromium on the resident gut microflora of Wistar rats.

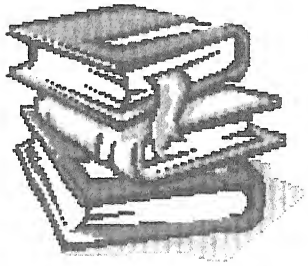
Aims and Objectives of the study:

Dengue is endemic all over India so is the occupational and non-occupational exposure to hexavalent chromium. The pathogenesis of dengue depends upon the immune response of the body. The intermediary products generated during reduction of chromium (VI) kill the target cells including leucocytes by apoptosis. This effect of chromium compromises the immune response of the host. It is, therefore, possible that the chromium toxicity may affect the disease process during dengue virus infection. There are no reports in literature on the outcome of dengue virus infections during chromium toxicity, therefore, the present study was undertaken.

Questions that have been addressed are:

1. What are the effects of subtoxic dose of Cr (VI) on the peripheral blood cells of mice during dengue virus infection?
2. Does Cr (VI) toxicity enhance the ill effects of dengue virus on macrophage functions and proliferative responses of lymphocytes?
3. Can the cells of immune system detoxify Cr (VI)?
4. Can the intestinal cells and the gut microflora help in detoxifying the ingested Cr(VI).

In the present study an attempt has been made to answer the above question using mice (which has been used most for dengue virus studies) and rats (used extensively for Cr (VI) studies). This may lead to better understanding of Cr (VI) toxicity and dengue virus infection.



Review of Literature

REVIEW OF LITERATURE

A number of trace metals are essential micronutrient and are required for various body functions and well being of the immune system. The deficiencies of trace metals and infectious diseases often coexist and exhibit complex interactions. Several trace metals such as selenium, zinc, copper, chromium and manganese *etc.* have immunomodulatory functions and thus influence the susceptibility, the course and the outcome of a host to various viral infections. Some trace metals inhibit virus replication in the host cells, thus showing antiviral activity. Many trace metals act as antioxidant or help such functions that not only regulate immune response of the host, but also may alter the genome of the viruses. The grave consequences of this may be the emergence of new infections. The trace metals, viruses and immune system interactions have been briefly reviewed in this article to highlight the importance of trace metal nutrition of host in not only optimizing immune response to infections, but also in preventing viral mutations which could increase the viral pathogenicity.

Chromium

Chromium is one of the naturally occurring heavy metal found in environment in many forms, the commonest stable oxidation states being trivalent Cr(III) and hexavalent Cr(VI). Cr(VI) compounds have been declared as a potent occupational carcinogen by IARC (1990) through epidemiological studies among workers in chrome plating, stainless-steel, and pigment industries. The reduction of Cr(VI) to Cr(III) results in the formation of reactive intermediates that contribute to the cytotoxicity, genotoxicity, and carcinogenicity of Cr(VI)-containing compounds.

The major non-occupational source of chromium for humans is food such as vegetables, meat *etc.* (Hertel, 1986). Other potential non-occupational

sources include urban air, hip or knee prostheses and cigarettes (U.S. EPA 1984). Cr(VI) is a widely used industrial chemical, extensively used in paints, metal finishes, steel including stainless steel manufacturing, alloy cast irons, chrome, and wood treatment. On the contrary, Cr(III) salts such as chromium polynicotinate, chromium chloride and chromium picolinate, are used as micronutrients and nutritional supplements, and have been demonstrated to exhibit a significant number of health benefits in animals and humans.

Chromium enters the body through the lungs, gastro-intestinal tract, and to a lower extent through skin (Hamilton and Wetterhahn, 1988). Inhalation is the most important route for occupational exposure (Hertel, 1986), where as non-occupational exposure occurs via ingestion of chromium containing food and water (Langard 1982; Pedersen, 1982). Regardless of route of exposure Cr(III) is poorly absorbed whereas Cr(VI) is more readily absorbed (Hamilton and Wetterhahn, 1988). Further, absorption of Cr(VI) is poorer by oral route therefore, it is not very toxic when introduced by oral route (Deflora, 1997). But chromium is very toxic by dermal and inhalation route and causes lung cancer, nasal irritation, nasal ulcer hypersensitivity reactions and contact dermatitis. Most of the chromium absorbed by inhalation exposure, in comparison to oral administration, is distributed in the lungs, liver, kidneys, RBC, plasma, spleen, bone-marrow (Langard, 1982). All the ingested Cr(VI) is reduced to Cr(III) before entering in the blood stream (Kerger *et al.*, 1996). The main routes for the excretion of chromium are via kidney/urine and the bile/feces (Guthrie, 1982; Langard, 1982). The localized amount of chromium varies from organ to organ.

Cr(III) is unable to enter cells but Cr(VI) enters into cells through membrane anionic transporters. Intracellular Cr(VI) is metabolically reduced to the ultimate Cr(III). Cr(VI) does not react with macromolecules such as DNA, RNA, proteins and lipids. However, both Cr(III) and the reductional intermediate Cr(V) are capable of co-ordinate covalent interactions with

macromolecules. Chromium plays an important role in glucose and cholesterol metabolism and is thus essential to man and animals (Schroeder *et al.*, 1962). Chromium is an essential nutrient required by the human body to promote the action of insulin in body tissues so that the body can use sugars, proteins and fats. Chromium picolinate has been used as nutritional supplement; it controls blood sugar in diabetes and may reduce cholesterol and blood pressure levels. Chromium increases insulin binding to cells, insulin receptor number and activates insulin receptor kinase leading to increased insulin sensitivity (Anderson, 2000). It also has beneficial effect on both muscle strength and body composition (Young, 1999). But high doses of chromium and long term exposure of it can give rise to various, cytotoxic and genotoxic reactions that affect the immune system of the body. However, the mechanism of the Cr(VI)-induced cytotoxicity is not entirely understood. A series of *in vitro* and *in vivo* studies have demonstrated that Cr(VI) induces an oxidative stress through enhanced production of reactive oxygen species (ROS) leading to genomic DNA damage and oxidative deterioration of lipids and proteins. A cascade of cellular events occur following Cr(VI)-induced oxidative stress including enhanced production of superoxide anion and hydroxyl radicals, increased lipid peroxidation and genomic DNA fragmentation, modulation of intracellular oxidized states, activation of protein kinase C, apoptotic cell death and altered gene expression.

Some of the important factors in determining the biological outcome of chromium exposure include the bioavailability, chemical speciation and solubility of chromium compounds, intracellular reduction, and interaction of chromium with DNA. At the genomic level, chromium genotoxicity manifests as gene mutations, several types of DNA lesions and inhibition of macromolecular synthesis. At the cellular level, chromium exposure may lead to cell cycle arrest, apoptosis, premature terminal growth arrest, or neoplastic transformation. chromium-induced DNA-DNA interstrand crosslinks, the

tumor suppressor gene p53 and oxidative processes are some of the major factors that may play a significant role in determining the cellular outcome in response to chromium exposure. Studies have utilized these approaches to understand the interrelationship between chromium-induced genotoxicity, apoptosis and effects on immune response.

Effects of Chromium on the Immune System

The immune system contributes to the maintenance of physiological integrity of the body by eliminating foreign material and infectious microbes, which enter it. This is mediated through nonspecific or specific acquired immunity which is a complicated process involving coordinated efforts of several types of cells and their secretary products, for example various antigen presenting cells, including macrophages and T and B lymphocytes. There are large numbers of biologically active compounds, which may have direct, primary or secondary effect on the immune system. The effect of chemicals including drugs, pesticides, hydrocarbons, heavy metals and many other organic and inorganic substances on the human immune system are of interest to pathologists, immunologists and toxicologists.

Various metals are responsible for many biochemical, immunological and physiological essential activities of the body as micronutrients. But some of them can give rise to disordered functions of the immune system resulting in increased susceptibility to infection, a variety of hypersensitivity reactions and autoimmune diseases and to neoplasia. Heavy metals are of significant importance in altering the immune response by immunostimulatory or immunosuppressive mode.

Effects of Chromium on lymphocytes:

The T and B lymphocytes are the effector cells of the immune system. The B lymphocytes react to an antigen by producing specific antibodies, while T lymphocytes help B cells in antibody production besides mediating the

cellular immune response. The effect of chromium on lymphocytes has been investigated in several studies. Borella *et al.* (1990) investigated the *in vitro* effect of toxic metals including Cr(III) and Cr(VI) on PHA-induced blastogenesis in human lymphocytes. Cr(VI) shows a biphasic pattern, with a stimulatory effect at the lowest concentrations tested and an inhibitory effect on at higher concentrations. No effect is observed when Cr(III) is added to the culture medium. Chromium has ability to enter the lymphocytes or to adhere to their surface, and the interaction appears to be quite stable. Faleiro *et al.* (1996) described the effect of cobalt-chromium-molybdenum (CoCrMo) disc samples on the CD3-mediated *in vitro* response of human peripheral blood T lymphocytes. Inhibition of lymphocyte proliferation is observed in the presence of CoCrMo disc samples. Ultrastructural studies using scanning electron microscopy revealed that the differences in the number of blast cells on CoCrMo discs from a 4 day culture are consistent with the results observed in the proliferation experiments, *i.e.* fewer blast cells are seen on the CoCrMo discs. The proliferation of both T and B cells and the production of immunoglobulin by lipopolysaccharide-stimulated B cells are significantly inhibited by cobalt-chromium particles at 3 weeks after intraperitoneal injection in mice (Wang *et al.*, 1997a). The cytokine release by lymphocytes, proliferation of T and B cells, and immunoglobulin production by B cells are also significantly inhibited by cobalt-chromium particles, as well as by cobalt and chromium ions *in vitro*, whereas these metals are not cytotoxic to murine lymphocytes *in vitro*. The data indicate that the metal-induced immunosuppression may be another important factor in the development of implant-associated infection in patients with a prosthesis (Wang *et al.*, 1997a).

On the other hand several studies have shown that chromium salts/alloys have no effect on cells of immune system. Yucesoy *et al.* (1999) investigated the immunotoxic effects of lead, cadmium, nickel and chromium on natural killer (NK) cell activity *in vitro*. None of the metal salts has any effect on NK

cell function. Similarly no effect is seen in stainless steel welders, exposed to chromium and nickel contained in welding fumes, for the kinetics of cell division in the culture of peripheral blood lymphocytes (Myslak and Kosmider,1997). Kulak and Arikan (1997) investigated immunoglobulin E (IgE) values, total erythrocyte, thrombocyte, leukocyte, lymphocyte, granulocyte and monocyte of dental laboratory technicians who use dental base metal alloys. They found that erythrocyte and thrombocyte values are significantly decreased but no differences are found in other blood parameters studied, including lymphocytes.

Effects of Chromium on Macrophages

Macrophages are among the cells of first line of defense due to their phagocytic, cytotoxic, and secretary activities. Any foreign material that enters body is phagocytosed by macrophage and digested. In this process the macrophage may also be damaged affecting its functions. Lungs have a rich supply of macrophage and have been used in various investigations. The inhalation of chromium does not affect lungs morphology, but macrophages are enlarged, multinucleated or vacuolated and accumulate in intraalveolar spaces as nodules. Chromium compounds also reduce the phagocytic activity. Higher dose of Cr(VI) depress the activity of alveolar macrophages and the humoral immune response, where as lower dose of Cr(VI) stimulates phagocytic activity of the alveolar macrophages and increases the humoral immune response (Glaser *et al.*, 1985). Macrophages can be induced to produce nitric oxide (NO), which is important for various functions. Tian and Lawrence (1996) studied the effect of various metals including chromium and reported that chromium does not modulate NO production by cytokine (IFN-γ, TNF-α)-stimulated murine macrophages. Chromium moderately suppresses inducible NO synthase (iNOS), which suggests that it may directly modify enzyme or cofactor activity. Overall, these observations provide additional insight into the means by which metals via inhibition or enhancement of NO production may

be pathogenic, by suppression of defense mechanisms or induction of hypersensitivity, respectively.

Howie *et al.* (1996) reviewed the literature on animal and cellular models used to study the response to cobalt-chrome alloy implants and wear and corrosion products. Animal studies show that in solid form cobalt-chrome alloy is relatively well tolerated. Injections of large numbers of particles in a single bolus lead to acute inflammation and necrosis, followed by a chronic inflammatory response. Macrophages are the predominant cell type and may persist in the tissues for years. Long term studies have failed to confirm the induction of tumors. *In vitro* studies confirm the toxic effects of cobalt-chrome alloy corrosion products and wear particles. *In vitro* studies show that cobalt-chrome alloy particles induce the release of inflammatory mediators from macrophages before causing cell death. These mediators have significant effects on osteoblast-like cells, as well as inducing bone resorption. The chromium orthophosphate corrosion product is a potent macrophage/monocyte activator and may contribute to macrophage-mediated osteolysis and aseptic loosening (Lee *et al.*, 1997).

Lee *et al.* (2000) evaluated dose-dependent effects of chromium chloride ($CrCl_3$) and chromium picolinate (CrP) for their glucose uptake, superoxide anion (O_2^-) production, activity of glucose-6-phosphate dehydrogenase, and phagocytosis of incubated pulmonary alveolar macrophages in medium in presence or absence of insulin. The phagocytosis of Escherichia coli by macrophages is enhanced significantly in medium containing $CrCl_3$ or CrP in the presence of insulin. The results suggest that the addition of $CrCl_3$ enhances directly the cellular activity of macrophages, whereas the effect of CrP requires the cooperative action of insulin in enhancing their glucose uptake and phagocytosis.

Gatta *et al.* (2001) studied the effects of dietary chromium yeast supplementation on the immune response of rainbow trout (*Oncorhynchus*

mykiss). A positive influence is observed on serum lysozyme activity in fish maintained on the high chromium diet. Significant differences are found in the level of respiratory burst elicited by macrophages of fish fed supplemented chromium after 3 and 6 weeks of feeding. Macrophages of fish receiving diets supplemented with chromium also have a greater ability to phagocytose yeast after 6 weeks than the control fish. The results of the study show that chromium yeast is able to modulate the immune response of rainbow trout, and this effect appears to be both dose- and time-dependent.

Jain and Kannan (2001) demonstrated that chromium supplementation inhibits TNF-α secretion in U937 monocytes cultured in high-glucose medium, which appears to be mediated by its antioxidative effect. This provides evidence for a novel molecular mechanism by which chromium supplementation may increase insulin sensitivity and glycemic control in diabetic patients.

Effects of Chromium on Cytokines

Cytokines are soluble glycoproteins released by cells of the immune system, which act nonenzymatically through specific receptors to regulate immune responses. They include a vast array of relatively low molecular weight, pharmacologically active proteins that are secreted by one cell for the purpose of altering either its own functions (autocrine effect) or those of adjacent cells (paracrine effect). Cytokines resemble hormones in that they act at low concentrations bound with high affinity to a specific receptor. In many instances, individual cytokines have multiple biological activities. Different cytokines can also have the same activity, which provides for functional redundancy within the inflammatory and immune systems. The biological effect of one cytokine is often modified or augmented by another. Because an interdigitating, redundant network of cytokines is involved in the production of most biological effects, it usually requires more than a single defect in the

network to alter drastically the outcome of the process. The synergistic and antagonistic effects that cytokines have on each other contribute significantly to the specificity of the immune response.

The effect of chromium on cytokines has been studied either by administration of chromium or implantation of prosthesis containing chromium-alloy. Myers *et al.* (1995) studied the effect of dietary chromium picolinate (CrP) and recombinant porcine growth hormone, somatotropin (rPST) administration on growth performance and cytokine production in Landrace-Poland China gilts. They showed that CrP-treated swine have very high IL-6 levels while no differences are seen in plasma IL-6 from pigs treated with the rPST and rPST+CrP. PBMC from CrP-treated animals produce more IL-2 than peripheral blood mononuclear cells from all other groups.

Shumilla *et al.* (1999) investigated the effects of Cr(VI) on basal and TNF-α-induced transcriptional competence of nuclear factor-Kappa$_B$ (NF-κ_B) in A549 human lung carcinoma cells. Pretreatment of A549 cells with nontoxic levels of Cr(VI) inhibited TNF-α-stimulated expression of the endogenous gene for IL-8 and of an NF-κ_B-driven luciferase gene construct, but not expression of urokinase, a gene with a more complex promoter. Chromium does not inhibit TNF-α-stimulated IκBα degradation or translocation of NF-κ_B-binding proteins to the nucleus. However, Cr(VI) pretreatments prevent TNF-α-stimulated interactions between the p65 subunit of NF-κB and the transcriptional cofactor cAMP-responsive element-binding protein-binding protein (CBP). These data indicate that nontoxic levels of Cr(VI) selectively inhibit NF-κB transcriptional competence by inhibiting interactions with coactivators of transcription rather than DNA binding.

Chromium exerts effect on immune function of women responsible for enhanced maturation of 'virgin' into "memory" lymphocyte in urban women who are exposed to chromium by vehicular traffic. Blood lymphocyte subsets

and serum IL-4 and IFN-γ levels of the non-atopic and non-symptomatic women are found to be similar. While serum IgE and *in vitro* production of IL-4 and INF-γ are higher spontaneously by mononuclear blood cells of atopic women who are exposed to an urban environment. Of the non-atopic subjects were significantly correlated with activated T, B, and NK HLA-DR + cells (Bascolo *et al.*, 2000). Differential leucocytes counts reveal that chromium causes a significant decrease in large and small lymphocytes, whereas neutrophils and thrombocytes are decreased.

Several studies have investigated the effect of prosthesis on the cytokine response of the body. Lee *et al.* (1997) showed that interfacial membrane mononuclear cells produce high levels of interleukin(IL)-1α, IL-1β, and prostaglandin E_2 (PGE_2) and express bone resorptive activities following stimulation with either titanium or chromium orthophosphate.

Horowitz *et al.* (1998) elucidated the mechanisms by which cobalt-chromium particulate wear debris contributes to the aseptic loosening of total joint prostheses. Incubation of macrophages with cobalt chromium leads to release of TNF-α and PGE_2, but do not lead to release of IL-1β or IL-6. Exposure of macrophages cocultured with osteoblasts to cobalt chromium also lead to significant release of TNF-α and PGE_2, but not to significant IL-6 or IL-1β production. The release of PGE_2 in the coculture system is greater than that detected when macrophages are exposed to cobalt chromium without the osteoblast contribution. Exposure of macrophages to cobalt chromium is toxic, as reflected by release of the intracellular enzyme lactate dehydrogenase. Macrophages play a role in the initiation of bone resorption at the interface through the phagocytosis of cobalt chromium particles and subsequent release of TNF-α and PGE_2. The presence of osteoblasts at the interface may be required for amplification of the inflammatory response and ultimately for bone resorption.

Prabhu *et al.* (1998) have examined a number of basic biological responses of the J774A.1 cell line, including cell proliferation, apoptosis, cytokines secreted into the culture supernatant (TNF-α, IL-1β, IL-6, and IL-12) and mRNA expression of the cytokines (TNF-α, IL-1β, IL-6, IFN-γ, M-CSF, and TGF-β) in response to cobalt-chrome alloy particles (CoCr). The results indicate that the relative contribution of CoCr particles in J774A.1 activation is negligible, and a change in metabolic activity of J774A.1 cells is observed only at higher concentrations of CoCr particles.

Granchi *et al.* (1998) determined if the serum levels of bone-resorbing cytokines (IL-1β, TNF-α, IL-6, GM-CSF) are altered in patients with aseptic loosening of a total hip prosthesis, and if such levels are influenced by the type of implant. The findings showed that the soluble receptor of IL-2 (sIL-2r) and TNF-α serum level do not change. The IL-6 level is not significantly altered but is higher in patients with TiAlV prostheses than in those with a CrCoMo implant and in patients with cemented prostheses. The IL-1β level is found to be higher in those with a TiAlV cemented prosthesis than in the control group. The GM-CSF level significantly increase in patients compared with healthy subjects, and it is higher in those with cemented than with uncemented implants. Only patients with cementless CrCoMo prostheses have levels of GM-CSF similar to those of the control group. The highest GM-CSF concentrations are observed in patients treated with non-steroidal anti-inflammatory drugs (NSAIDs) in the last months before revision. In addition, when massive osteolysis is observed, the level of GM-CSF tends to decrease to that of the control group. They further evaluated of the release of bone-resorbing cytokines in peripheral blood mononuclear cells (PBMC) of patients with aseptic loosening of Co-Cr hip prostheses (Granchi *et al.*, 1999). TNF-α, IL-6, and GM-CSF are measured in both unstimulated and PHA-stimulated PBMC, and in PBMC cultured in the presence of chromium and cobalt extracts. The proportion of lymphocyte, monocyte and lymphocyte

subpopulation in cultured PBMC do not differ in patients and the control group. In unstimulated PBMC the release of TNF is significantly higher in patients than in the control group, while IL-6 is significantly decreased and no change is observed for GM-CSF. When the PBMC are challenged with chromium extract, all the 'index of cytokine release' results higher in patients than in the control group; cobalt extract increased both the TNF and GM-CSF index, but not the index of IL-6 release. Metal concentrations in serum from patients are significantly increased and correlate with the TNF release in PBMC stimulated with both metal extract. The results suggest that a CoCr-implant releases a large amount of metal ions which could mediate the priming or the renewal of a cell-mediated hypersensitivity reaction. The prevalence of circulating lymphocytes responsible for the delayed hypersensitivity, namely Thl, would justify both the significant increase of TNF and the significant decrease of IL-6 in unstimulated PBMC of patients, as well as the significant increase of the 'index of cytokine release' after the challenge with metal ions.

Wang *et al.* (1997a) investigated whether prosthetic metals adversely affect immune responses and the release of immunoregulatory cytokines *in vivo* and *in vitro*. Cobalt-chromium alloy were injected into the peritoneal cavity of female mice. The release of IL-2 and IL-4 is significantly inhibited by cobalt-chromium particles after 3 weeks while the release of IFN-γ is significantly inhibited only at 12 weeks (Wang *et al.*, 1997a). They have further reported that chromium significantly enhanced the release of IL-1β and TNF-α by lipopolysaccharide stimulated human osteogenic sarcoma cells, whereas the release of TGF-β1 and IL-6 is not altered. This indicates that the metal induced disregulation of cytokine release and osteoblast dysfunction may play an important role in the induction of osteolysis in patients with total joint arthroplasties (Wang *et al.*,1997b).

Effects of Chromium on Immune Response

Burton *et al.* (1993) studied the effects of supplemental dietary chromium on immune responses of dairy cows subjected to physical and metabolic stresses associated with late pregnancy, calving, early lactation, and peak milk yield. They showed that supplemental chromium causes elevated antiovalbumim (OVA)-antibody responses and mitogen-stimulated blastogenic responses of peripheral blood mononuclear cells (PBMC), is associated with lowered OVA-stimulated blastogenic responses of PBMC, and has no overall effect on antibody responses to horse RBC relative to responses of control cows. These results indicate that supplemental chromium can alter specific immune responses of stressed cattle.

Another study was undertaken (Chang *et al.*, 1996a) to determine the effects of supplemental dietary chromium on health status and mastitis-related parameters, as well as neutrophil phagocytic activity of dairy cows during late pregnancy and early lactation. In addition, possible interactions and involvements of chromium with insulin, cortisol, somatotropin (rBST) and insulin-like growth factor-1 (IGF-1) were directly investigated *in vitro* based on blastogenic responses using peripheral blood lymphocytes (PBL) of eight cows fed the control diet. Forty pregnant Holsteins, 18 primiparous and 22 multiparous, at week 6 before the expected calving dates were randomly assigned to treatments: control and supplemental chelated chromium (0.5 ppm) in the diet. Supplemental chromium has no effect on health status of cows during late pregnancy and early lactation. Supplemental chromium also did not affect neutrophil phagocytic function of cows from 6 weeks prepartum to 6 weeks postpartum. However, in the *in vitro* study of PBL (from control animals, not fed chromium diet) blastogenesis with addition of insulin or cortisol, insulin and cortisol enhanced or had no effect on PBL proliferations with or without Concanavalin A (Con A) simulation. However, further chromium addition in the culture medium containing supplemental insulin or

cortisol, particularly CrCl3, additively increased BPL blastogenic activities with or without Con A stimulation. Conversely, addition of rBST or IFG-1 in the culture medium enhanced PBL proliferation, but addition of chromium gave no additional effect. These results indicate that supplemental chromium had no beneficial effect on health status, mastitis-related parameters or neutrophil phagocytic activity of dairy cows (Chang *et al.*, 1996). van de Ligt *et al.* (2002a) have studied the effect of chromium tripicolinate supplementation of diet on porcine immune response during postweaning period and found no effect on the performance and immune status. Further, the effects of chromium tripicolinate supplementation on immune response in sows and their offspring during the periparturient and neonatal period has been studied. The supplementation has minimal effects on humoral antibody response of the dam or its transfer to the neonate (van de Ligt *et al.*, 2002b)

Khangarot *et al.* (1999) studied the effects of subtoxic levels of chromium on humoral and cell-mediated immune responses, blood parameters, susceptibility to bacterial (*Aeromonas hydrophila*) infection, and macrophage activity in the freshwater air-breathing Asian catfish, *Saccobranchus fossilis*, during a 28-day exposure were examined by a static bioassay test procedure. Fish exposed to chromium have lower spleen weight, lower antibody titer, reduced numbers of splenic and kidney plaque-forming cells, and higher counts of splenic lymphocytes but reduced counts of kidney cells when compared with the control group. A dose-dependent decrease in red blood cell counts, hemoglobin content, and packed cell volume are observed. Differential leukocyte counts revealed that chromium exposure causes a significant decrease in large and small lymphocytes, whereas neutrophils and thrombocytes increase. Con A induced proliferation of splenic and pronephric lymphocytes is decreased. The eye-allograft rejection time, as a parameter of cell-mediated immunity, is increased. Fish exposed to chromium for 28 days exhibit higher susceptibility to *A. hydrophila* infection than the control fish.

The phagocytic activity of splenic and pronephros macrophages is significantly decreased. das Neves *et al.* (2001) investigated the morphology of mouse splenic cells after short-term exposure to Cr(VI) and the effects were studied 24 and 48 h after the injections. Histological results show a time-dependent effect of Cr(VI) on splenic cells. Changes include enlargement of the capsule and depletion of the red pulp cells, accompanied by an increase in macrophages, 24 h after injection. Partial restoration of red pulp is noted after 48 h.

Arunkumar *et al.* (2000) investigated the effect of Cr(III) and Cr(VI) in the African mouth breeder *Oreochromis mossambicus* (Peters), with reference to the humoral immune response and lymphoid cells/organs. Groups of fishes were injected intraperitoneally with Cr(III) and Cr(VI) and subsequently immunized with bovine serum albumin. Both forms of chromium suppress the antibody response, with Cr(VI) being more suppressive than Cr(III). Reduction in spleen weight, splenocyte number and the percentage of blood lymphocytes is observed following administration of both forms of chromium.

An early study done on human cells is of Borella *et al.* (1993) who studied the effects of Cr(VI) and other metals in cultured human lymphocytes. Both the proliferative response and the generation of antibodies were evaluated, and the metal accumulation in the cells was measured. It is found that chromium induces reductions in both blastogenesis and Ig production in relation to its capability to enter the cells.

Chromium-induced hypersensitivity reactions

When a metal ion enters in a biological system it does not travel as free metal but may bind with autologous proteins and render them immunogenic, thus inducing hypersensitivity reactions. Chromium induces two types of hypersensitivity reactions, Type I- Anaphylactic type and Type II- the Delayed type hypersensitivity (Kazantis, 1990; Avnstorp *et al.*, 1990). The Type I hypersensitivity reaction is mediated by IgE antibody bound to mast cells that

release vasoactive mediators on contact with specific antigen. The Type IV hypersensitivity reaction is mediated by helper T (Th)-1 cells.

Chromium is the one of the most common skin sensitizer in the general population. Such exposure involves handling cement, tanning of leather, chromium platting etc. Cr(VI) readily transverses cell membrane and undergoes intracellular reduction to Cr(III) by forming a conjugate with proteins to act as complete antigen. Circulating antibodies against Cr(III) but not against Cr(VI) have been identified in sensitized animals (Novey *et al.*, 1983). Dermal exposure of chromium produces irritant and allergic contact dermatitis (Brugnzeet *et al.*, 1988; Polak, 1983). Irritant dermatitis causes due to cytotoxic effect of chromium whereas allergic contact dermatitis is an inflammatory response mediated by immune system. It is observed that keratinocytes are the first target cells affected by chromium, causes contact dermatitis, these cells can be directly activated through the expression of the membrane antigen ICAM-I, a ligand of the leucocytes antigen LFA-I and the production of cytokines including a significant release of TNF-α. These finding indicate that chromium may be able to induce an aggressive cellular effect and may play a major role in keratinocyte activation during contact dermatitis (Gueniche *et al.*, 1994). Development of allergic contact dermatitis by exposure to chromium has been reported in several studies (Estlander *et al.*, 2000; Kanerva *et al.*, 2000; Kvitko, 2001; Silvestre *et al.*, 2001). Thomas *et al.* (2000) reported on a 37-year-old man who developed an aseptic intolerance reaction to a chromium-cobalt alloy. Skin testing gave a delayed-type reaction to dichromate. Immunohistology reveal a monocytic and dense T-cell infiltrate. The latter, instead of being random, show an oligoclonal T-cell receptor rearrangement. The actual tissue mRNA expression for IL-4, IL-6, and IFN-α could be visualized by RT-PCR. This indicate a TH1-type mediator expression (IL-6 and IFN-α, but not IL-4).

Chromium may also be less commonly involved in immediate or type I hypersensitivity reactions. Cases of asthma with an immediate or delayed response has been observed following occupational exposure to chromium (Keskinen *et al.*, 1980; Novey *et al.*, 1983; Park *et al.*, 1994; Leroyer *et al.*, 1998; Sastre *et al.*, 2001). Hassmanova *et al.* (2000) have studied occupational diseases caused by chromium and its compounds. They have reported perforations of the nasal septum, bronchial asthma, allergic rhinitis, and contact allergic eczemas, and an exceptional finding of a chromium ulcer (pigeonneaux) on the lower extremity of a builder.

Chromium-induced cell death

Chromium is known to have cytotoxic effects on cells. Rajaram *et al.* (1995) reported that chromium induces abnormalities in human lymphocyte which are suggestive of apoptosis, a programmed cell death. Vasant *et al.* (2001) have shown that apoptosis is the mode of cell death of human lymphocytes in the presence of both Cr(V) and Cr(VI). Pretreatment of cells with antioxidants before exposure to chromium(V) complexes reverses apoptosis partially. Possibility for the formation and implication of reactive oxygen species in Cr(V)-induced apoptosis of human lymphocyte cells has also been indicated. Hexavalent chromium induces apoptosis in CHO cells also (Shimada *et al.*, 1998). The studies of Carlisle *et al.* (2000) show that chromium-induced apoptosis of normal diploid human lung fibroblasts is p53 dependent. These studies indicate that chromium induces cell death by apoptosis.

Mechanisms of action of chromium on cells of immune system

Easy availability, convenient to handle and maintain *in vitro* in culture make lymphocyte and macrophage ideal cells to study the mechanism of cytotoxicity and genotoxicity and thus, the mechanism of effects on immune functions of body. Initial studies used DNA strand breaks and sister-chromatid exchange (SCE) in blood lymphocytes to investigate the toxicity of chromium.

Gao *et al.* (1992) incubated human lymphocytes with Cr(VI) at 37°C for 3 h and showed a dose-dependent increase in DNA strand breaks without concurrent cytotoxicity. In contrast, Cr(III) fails to induce DNA strand breaks at sub-cytotoxic concentrations. Gennart *et al.* (1993) determined SCE in blood lymphocytes in 26 male workers occupationally exposed to chromium, cobalt and nickel dust and in 25 controls matched for age and smoking habits. An analysis of variance on the SCE rank values reveal that both exposure status (exposed persons vs. controls) and smoking habits (smokers and former smokers vs. never smokers) have a statistically significant effect.

Katsifis *et al.* (1996) and Lai *et al.* (1998) compared the effect of chromium and Ni-Cr on SCE in lymphocytes to obtain an understanding of the mutagenic effect of Cr(VI) in humans. The data indicate that antagonism may occur when human lymphocytes are exposed simultaneously to Ni(II) and Cr(VI), suggesting an explanation for epidemiological studies reporting conflicting results for cytogenetic effects in lymphocytes of workers exposed to chromium and nickel. On the other hand additive damaging effect of chromium and curcumin on DNA of human lymphocytes and gastric mucosa (GM) has been reported by Blasiak *et al.* (1999) by using the alkaline single cell gel electrophoresis (comet assay). The study clearly demonstrates that curcumin does not inhibit DNA damaging action of Cr(VI) in human lymphocytes and GM cells. Moreover, curcumin itself can damage DNA of these cells and the total effect of chromium and curcumin is additive.

Blasiak and Kowalik (2000) compared the effects of Cr(III) and Cr(VI) on the DNA damage in human lymphocytes using the comet assay. The effect is dose-dependent. Treated cells recover within a 120-min incubation. Cr(III) causes greater DNA migration than Cr(VI). Catalase, an enzyme inactivating hydrogen peroxide, decreases the extent of DNA damage induced by Cr(VI) but not the one induced by Cr(III). Lymphocytes exposed to Cr(VI) and treated with endonuclease III, which recognizes oxidized pyrimidines, displayed

greater extent of DNA damage than those not treated with the enzyme. Such an effect is not observed when Cr(III) is tested. The results obtained suggest that reactive oxygen species and hydrogen peroxide may be involved in the formation of DNA lesions byCr(VI). The comet assay did not indicate the involvement of oxidative mechanisms in the DNA-damaging activity of Cr(III) and due to its binding to cellular ligands it may play a role in its genotoxicity. Trzeciak et al (2000) studied chromium-introduced damage to DNA both in the GM cells and lymphocytes by comet assay. The effect induced by K2Cr2O7 in GM cells is similar to that seen in the lymphocytes.

Recent *in vitro* and *in vivo* studies have investigated the effect of Cr(VI) on human PBMC, chronic myelogenous leukemic K562 cells and J774A.1 murine macrophage cells and on female C57BL/6Ntac and p53-deficient C57BL/6TSG p53 mice using comet assay, laser scanning confocal microscopy and flow cytometry *etc.*(Bagchi *et al.*, 2001; Stohs *et al.*, 2001). The findings demonstrate that chromium induces an oxidative stress that results in oxidative deterioration of biological macromolecules. Chromium undergoes redox cycling, resulting in enhanced production of reactive oxygen species such as superoxide ion, hydroxyl radicals, and hydrogen peroxide. These reactive oxygen species result in increased lipid peroxidation, enhanced excretion of urinary lipid metabolites, modulation of intracellular oxidized states, DNA damage, membrane damage, altered gene expression, and apoptosis. Enhanced production of nuclear factor-κB and activation of protein kinase C occur. Furthermore, the p53 tumor suppressor gene is involved in the cascade of events associated with the toxicities of these cations. Taken together, oxidative stress and oxidative tissue damage, and a cascade of cellular events including modulation of apoptotic regulatory gene p53 are involved in Cr(VI)-induced toxicity and carcinogenesis (Bagchi *et al.*, 2001; Stohs *et al.*, 2001).

Dengue Virus

Dengue is a mosquito-borne virus infection which is found in tropical and sub-tropical regions around the world, predominantly in urban and semi-

urban and now in rural areas also. Dengue is caused by four distinct viruses (type 1 to 4) that are closely related antigenically. Humans are the main amplifying host of the virus. Recovery from infection by one provides lifelong immunity against that serotype but confers only partial and transient protection against subsequent infection by the other three. It has been suggested that sequential infection increases the risk of more serious disease resulting in dengue haemorrhagic fever (DHF).

Before 1970 only nine countries had experienced DHF epidemics. The prevalence of dengue has grown dramatically in recent decades. The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia and the Western Pacific. Southeast Asia and the Western Pacific are most seriously affected. Some 2500 million people — two fifths of the world's population — are now at risk from dengue. WHO currently estimates there may be 50 million cases of dengue infection worldwide every year. During epidemics of dengue, attack rates among susceptibles are 40 to 90%. An estimated 500 000 cases of DHF require hospitalization each year, of whom a very large proportion are children (Halstead, 2002; Agarwal *et al.*, 1999). The year 2001 witnessed unprecedented global dengue epidemic activity in the American hemisphere, the Pacific islands and continental Asia. During 2002, more than 30 Latin American countries reported over 1000000 DF cases with large number of DHF cases. This has been followed by extensive epidemics of DHF in several parts of India during 2003.

The Indian encounter with dengue and DHF is interesting and intriguing. The first major epidemic illness compatible clinically with dengue occurred in Madras in 1780 and later on spread to all over the country. The dengue virus was first isolated in Japan in 1943 but the one isolated at Calcutta in 1944 from the blood of US soldiers was considered first report for a long time. DHF, a potentially lethal complication of dengue virus infection, was first recognized in the 1950s during the dengue epidemics in the Philippines and Thailand and quickly spread to other parts of the World (Gubler *et al.*, 1997). Today DHF

affects most Asian countries and has become a leading cause of hospitalization and death among children in several of them. The risk factors for DHF are infestation with *Aedes* mosquito, hot and humid climate enhancing mosquito breeding, mosquito density, and presence of all the four serotype of the dengue virus with the secondary infection in the host, the water storage pattern in the houses, population density and large movement of people towards urban areas. While DHF was present in the neighboring countries for a long time it was not known why it is not coming to India as all the risk factors are present in this country. When the first extensive epidemic of DHF occurred during 1996 in the Northern India there was no clue of its emergence.

The 1996 epidemic in India was mainly due to dengue type 2 virus while the 2003 epidemic appears to be mainly due to dengue type 3 viruses. Over the past two decades, dengue virus type 3 (DV-3) has caused unexpected epidemics of DHF in Sri Lanka, East Africa and Latin America. Isolates from these geographically distant epidemics are closely related and belong to DV-3, subtype III, which originated in the Indian subcontinent. The emergence of DHF in Sri Lanka in 1989 correlated with the appearance there of a new DV-3, subtype III variant. This variant likely spread from the Indian subcontinent into Africa in the 1980s and from Africa into Latin America in the mid-1990s. DV-3, subtype III isolates from mild and severe disease outbreaks form genetically distinct groups (Messer *et al.*, 2003) which suggest a role for viral genetics in DHF. There is need to genotype the dengue viruses isolated from the different parts of this country and study their relationship. At the same time human leukocyte antigen alleles correlate with both protection and susceptibility to dengue hemorrhagic fever and dengue shock syndrome; studies in Haiti suggest that blacks have a gene providing nearly complete protection against severe dengue illness.

Early diagnosis of dengue virus infection is important and can be established with easily available laboratory tests. But what is the value and appropriate use of the tourniquet test in dengue? A negative test does not rule

out dengue infection, a positive test should be followed by close surveillance for early signs of DHF. Low platelet counts do not predict clinically significant bleeding in dengue. It follows that platelet or blood transfusions should not be administered based upon platelet count alone. DHF or dengue shock syndrome cases frequently have compensated consumptive coagulopathy that seldom requires treatment. Bleeding is most likely caused by activated platelets resulting from damaged capillary endothelium. There is no specific treatment for dengue fever. However, careful clinical management frequently saves the lives of DHF patients. With appropriate intensive supportive therapy, mortality may be reduced to less than 1%. Maintenance of the circulating fluid volume is the central feature of DHF case management. DHF and dengue shock syndrome can be safely treated with just normal saline. Colloids should be immediately given to children presenting with a pulse pressure at or below 10 mmHg.

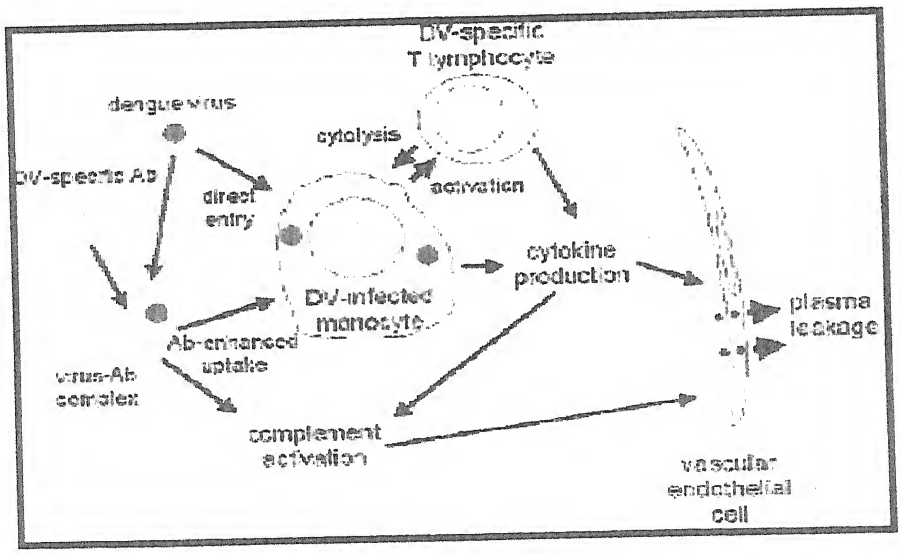


Figure II.1: Schematic presentation for the mechanism of pathogenesis during dengue virus infection.

Viral virulence and immune responses have been considered as two major factors responsible for the pathogenesis of DHF. Virological studies are attempting to define the molecular basis of viral virulence. The immunopathological mechanisms appear to include a complex series of immune responses (Fig. II.1). A rapid increase in the levels of cytokines and

chemical mediators apparently plays a key role in inducing plasma leakage, shock and haemorrhagic manifestations (Chaturvedi *et al.*, 1999b; 2000). It is likely that the entire process is initiated by infection with a so-called virulent dengue virus, often with the help of enhancing antibodies in secondary infection, and then triggered by rapidly elevated cytokines and chemical mediators produced by intense immune activation. The co-circulation of multiple dengue virus serotypes in the same region has been a common knowledge in several countries including India. Using a modified multiplex reverse transcription-polymerase chain reaction assay, concurrent infections by two different serotypes of dengue virus have been shown to occur in the same patient. Such concurrent infections by two dengue viruses may also increase the severity of the disease. However, understanding of the DHF pathogenesis is not complete.

Vaccine development for dengue and DHF is difficult because any of four different viruses may cause disease, and because protection against only one or two dengue viruses could actually increase the risk of more serious disease. However, effort is being made in the development of vaccines that may protect against all four dengue viruses. Several promising vaccine candidates in the form of live attenuated and chimeric vaccines have been developed and are currently in human clinical trials. However, significant practical, logistic, and scientific challenges remain before these vaccines can widely and safely be applied to vulnerable populations.

At present, the only method of controlling or preventing dengue and DHF is to combat the vector mosquitoes. *Aedes aegypti* breeds primarily in man-made containers like earthenware jars, metal drums and concrete cisterns used for domestic water storage, as well as discarded plastic food containers, used automobile tyres and other items that collect rainwater. It can also breed extensively in natural habitats such as tree holes and leaf axils (Fig. II.2). In recent years, *Aedes albopictus*, a secondary dengue vector in Asia, has become

established in the United States, several Latin American and Caribbean countries, in parts of Europe and in one African country. The rapid geographic spread of this species has been largely attributed to the international trade in used tyres.

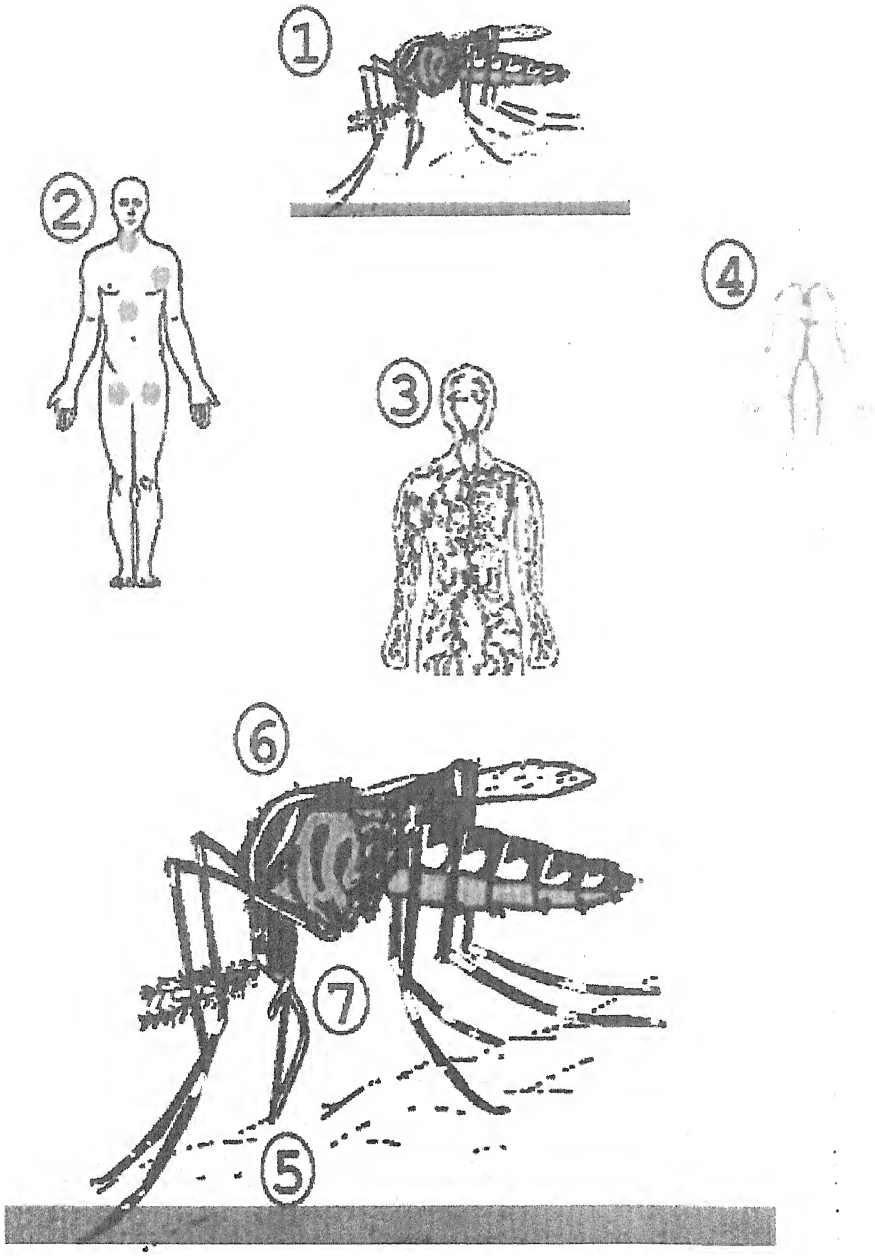


Fig. II.2: 1.Virus transmitted to human in mosquito saliva, 2. Vrus replicates in Target organs, 3. Virus infects white blood cells and lymphatic tissues, 4.Virus released anf circulates in blood, 5. Second mosquito ingest virus with blood, 6. Virus replicates in mosquito midgut and other organs, infects salivary glands, 7. Virus replicates in salivary glands.

Dengue continues to be a Global challenge because the pathogenesis of DHF is not fully understood, there is no immediate prospect of a vaccine and the mosquito control measures are inadequate. The wide spread DHF epidemics during 2003 reinforces the belief that DHF has come to stay in this country and will continue to spread to newer areas unless vector control measures are taken up on war footings.

Dengue virus-induced Cytotoxic Factor

During dengue virus infection the CD4+ T cells produce a unique cytokine, Cytotoxic Factor (CF), in mice (mCF) and its homologue in man (hCF). The amino-terminal sequence of mCF has no homology with any of the known proteins or cytokines. mCF and hCF appear to be pathogenesis-related proteins, capable of reproducing DHF-like pathological lesions in mice, such as increased capillary permeability, cerebral edema, and blood leukocyte changes (Chaturvedi *et al.*, 1991, 1997; Mukerjee and Chaturvedi, 1995; Mukerjee *et al.*, 1997). During an extensive epidemic of DHF in Northern India during 1996, the presence of hCF was shown in 90% of the 333 patients with peak amounts in the most severe patients with DHF grade IV. Further, *ex vivo* culture of peripheral blood mononuclear cells of such patients showed production of hCF by CD4+ T cells (Agarwal *et al.*, 1998a, 1998b). The production of mCF/hCF precedes the clinical illness in mice and man (Agarwal *et al.*, 1998a; Mukerjee and Chaturvedi, 1995; Mukerjee *et al.*, 1997; Chaturvedi *et al.*, 1999).

Recently a mechanism has been proposed to explain the pathogenesis of DHF in which hCF plays a key role (Chaturvedi *et al.*, 2000). Dengue virus replicates in macrophages and induces quickly the CD4+ T cells to produce hCF. hCF induces macrophages to produce free radicals, nitrite, reactive oxygen and peroxynitrite (Misra *et al.*, 1996, 1998). The free radicals, besides killing the target cells by apoptosis also directly upregulate production of

proinflammatory cytokines IL-1α, TNF-α, IL-8, and hydrogen peroxide in macrophages. The change in relative levels of IL-12 and TGF-α shifts a Th1-dominant response to a Th2-biased response resulting in an exacerbation of dengue disease and death of patients (Chaturvedi *et al.*, 1999b). The vascular permeability is increased due to the combined effect of histamine, free radicals, proinflammatory cytokines and the products of the complement pathway *etc.* Thus the key player appears to be hCF, but what regulates its activity is not known (Chaturvedi *et al.*, 2000).

Effects of Chromium on Viral Infections

Effects of Chromium on Sandfly Fever Virus Infection

Acute infection with Sandfly fever virus reduces availability of circulating chromium, which may contribute to the altered glucose metabolism characteristic of acute infection even in presence of elevated insulin levels and other hormonal changes (Pekarek *et al.*, 1975).

Effects of Chromium on Bovine HSV-1 Infection

Arthington *et al.* (1997) has observed that chromium-supplementation do not alter stress responses of calves experimentally inoculated with bovine HSV-1. Rectal temperatures are elevated but are not affected by chromium treatment. Secretion of ACTH, cortisol or plasma TNF-α is not affected by chromium treatment, although clear circadian variation in ACTH and cortisol occurs. No difference is detected in the concentrations of trace minerals excreted daily in the urine, lymphocyte proliferative response to mitogen stimulation and neutrophil bactericidal function. The acute phase proteins ceruloplasmin and fibrinogen also are not affected by treatment or viral challenge. However, it will be interesting to study the effects of chromium on human herpes virus infection.

Effects of Chromium on Infectious Bovine Rhinotrachitis virus (IBRV)

Kegley *et al.* (1997) have reported that when steers are inoculated with IBRV intranasally, average daily gain from day 0 to 80 is increased by supplemental chromium. Transportation of steers increased the ratio of neutrophils to lymphocytes. Supplemental chromium did not affect rectal temperature after the IBRV challenge or the antibody response to IBRV or porcine red blood cells. Supplemental chromium did not affect any of the immune response that was measured. According to another report, supplemental chromium has no effect on antibody response to IBRV, parainfluenza 3, bovine respiratory syncytial virus. However, it enhanced the antibody titres of calves in response to the bovine viral diarrhoea vaccine. These findings suggest that supplemental chromium can enhance humoral response of market-transmit stressed calves but its enhancement on vaccine efficacy is antigen dependent and variable.

Role of Gastro-intestinal tract in detoxification of Chromium:

The gastro-intestinal tract (GIT) is exposed to different environmental pollutants that contaminate food and water. These include metals that may have toxic effects on body. Many metals have no known biological function and some of these are capable of disrupting essential physiological processes. Examples of this are arsenic, cadmium, lead, chromium and mercury. The toxic effects of most metals can be traced to their ability to disrupt the function of essential biological molecules, such as proteins, enzymes and DNA. In some cases this involves displacing chemically related metal ions that are required for important biological functions such as cell growth, division and repair.

Certain heavy metals form very stable and long-lasting complexes with sulfur in biological molecules, which can disrupt their biological function. In some cases these metals may be concentrated at higher levels of the food chain. Body has developed various mechanisms to detoxify the toxic substances,

including the metals. In these regards, the cells and the secretions of GIT play an important role. The cells have evolved a complex network of metal trafficking pathways. The object of such pathways is to prevent accumulation of the metal in the freely reactive form (metal detoxification pathways) and to ensure proper delivery of the ion to target (Luk *et al.*, 2003). In recent times, microbes have been shown to reduce a wide range of toxic metals, for example Cr(VI), mercury, cobalt, lead and arsenic. Under certain conditions, microbial metal reduction can mobilize toxic metals with potentially calamitous effects on human health. Lloyd (2003) has discussed the role of microbes in reducing different metals and its impact on the environment. The resident gut microflora may also have a significant role to play in detoxification and elimination of the harmful metals from the body.

Gut Microflora: A large number of bacteria belonging to 300–500 different species live and grow in the human intestine as symbionts (Simon and Gorbach, 1984; Borriello, 1986). Table II.1 presents a list of common resident bacteria found at different locations in the GIT. The stomach, duodenum (0-10^4/g of the luminal contents) and small intestine (10^5-10^6/g) contain smaller number of bacteria adhering to the epithelia and some other bacteria in transit. This may be because of the composition of the luminal fluid containing acid, bile, and pancreatic secretion, which kills most ingested microorganisms. On the other hand, the large intestine contains a complex and dynamic microbial population with high densities of living bacteria. The luminal contents may have up to 10^{11} or 10^{12} bacteria/g. Some of these bacteria are potential pathogens and can be a source of infection and sepsis under certain conditions, for example when the integrity of the bowel barrier is physically or functionally broken down. A constant interaction between the host and the microbes provide important health benefits to the human host (Salminen *et al.*, 1998).

Microbes start colonization of the gastrointestinal tract soon after birth and continue throughout the life. The environmental factors have a major role

in determining the extent and type of colonization, for example, differences exist between people in developed countries and those in developing countries (Simhon *et al.*, 1982; Adlerberth, 1991). Some bacteria can modulate expression of genes in host epithelial cells (Hooper *et al.*, 2001), thus creating a favourable habitat for themselves, and can prevent growth of other bacteria introduced later. The initial colonization is therefore very relevant to the final composition of the permanent flora in adults. Studies have shown that anaerobic bacteria outnumber aerobic bacteria by a factor of 100–1000. The predominant genera in human beings are *bacteroides, bifidobacterium, eubacterium, clostridium, peptococcus, peptostreptococcus,* and *ruminococcus etc.* (Simon and Gorbach, 1984; Salminen *et al.*, 1998) followed by aerobes (facultative anaerobes) such as *Escherichia, enterobacter, enterococcus, klebsiella, lactobacillus, proteus* etc. (Table II.1). Every individual has several hundreds of species, with a particular combination that is distinct from that found in other individuals. The species vary greatly between individuals (Simon and Gorbach, 1984; Moore and Moore, 1995).

Table II.1: Common resident gut microflora

Part of GIT	Some Common Resident Bacteria
Mouth and oropharynx	Streptococcus viridians, Streptococcus pneumoniae, Beta- haemolytic streptococci, coagulase-negative Staphylococci , Veillonella spp., Fusobacterium spp., Treponema spp., Porphyromonas spp., Prevotella spp., Neisseria spp. and Branhamella catarrhalis, Candida spp., Haemophilus spp., Diphtheroids, Actinomyces spp.,Staphylococcus aureus, Eikenella corrodens
Stomach	Streptococcus, Staphylococcus, Lactobacillus, Peptostreptococcus
Small intestines	Lactobacillus spp., Bacteroides spp., Clostridium spp., Mycobacterium spp., Enterococci, bacteria of Enterobacteriaceae,
Large intestines	Bacteroides spp., Fusobacterium spp., Clostridium spp., Peptostreptococcus spp., Escherichia coli, Klebsiella spp., Proteus spp., Lactobacillus spp., Enterococci, Streptococci spp., Pseudomonas spp., Acinetobacter spp., coagulase-negative Staphylococci, Staphylococcus aureus, Mycobacterium spp., Actinomyces spp., Bifidobacterium bifidum, Enterobacter spp., Peptococcus spp., Methanogens (Archaea), Salmonella spp.

Gut Microflora as Probiotics and prebiotics: Probiotic is a bacterium that provides specific health benefits when consumed as a food component or supplement. They are living microorganisms that upon ingestion in specific numbers exert health benefits beyond those of inherent basic nutrition (Guarner and Schaafsma, 1998). According to this definition, probiotics do not necessarily colonize the human intestine. The effect of a bacterium is strain specific and cannot be extrapolated even to other strains of the same species. The prebiotics are non-digestible food ingredients that benefit the host by selectively stimulating growth, or activity, or both, of one or a restricted number of bacteria in the colon (Gibson and Roberfroid, 1995).

Reduction of Cr (VI) to Cr (III) by Microbes: The presence of chromate in the environment inhibits most microorganisms but also promotes the selection of resistant bacteria (Olukoya *et al.*, 1997). Cr (VI) compounds are markedly effective than those of Cr (III) due to their high solubility in water, rapid permeability through biological membranes and subsequent interaction with intracellular protein and nucleic acids. The bacteria present naturally in soil and water-bodies are exposed to Cr through contamination with industrial effluents, especially from tannery etc. The processes by which the microorganisms interact with the toxic metals enabling their removal/and recovery are biosorption, bioaccumulation and enzymatic reduction. Microorganisms have evolved resistance mechanism to select resistant variants to deal with metal toxicity as the result of exposure to metal contaminated environments, which cause co-incidental selection for resistant factors for antibiotics and heavy metals.

There are evidences for possible links between heavy metal and antibiotic resistance in a bacteria because these traits are generally associated with transmissible plasmids and the genes are frequently found on the same plasmid (Pathak and Gopat, 1994; Ramteke, 1997). Bacterial resistance to chromate can be due to chromosomal mutation or is plasmid-borne (Silver and

Misra, 1988). Many bacteria belonging to genera *Pseudomonas, Aeromonas, Enterobacter, Escharichia Bacillus, Streptomyces, etc.* can reduce Cr (VI) to Cr (III). In each case, the plasmid bearing strains is approximately 10-fold more resistant to chromate than is in the plasmid less strain (Cervantes and Silver, 1988). Under environmental conditions of metal stress, such metal and antibiotic resistant population adopts faster by the spread of R-factors than by mutation and natural selection thus leading to a very rapid increase in their numbers (Bhattacherjee *et al.*, 1998). Microbial resistance to metal ions and antibiotics is a potential health hazard.

Some bacteria present in water and soil develop resistance to chromium on exposure to Cr-containing effluent in their environment (Ohtake *et al.*, 1987; Wang *et al.*, 1990; Olukoya *et al.*, 1997; Viti *et al.*, 2003). These bacteria reduce Cr (VI) into Cr (III) and minimize the adverse effects of Cr (VI) on their growth (Yamamoto *et al.*, 1993). Further, some of the bacteria bioaccumulate large quantity of Cr and bring down the residual concentration of Cr (VI) in 24 h (Srinath *et al.*, 2002). Several studies have been done to investigate the effect of Cr on soil and water bacteria resistant to chromium (Ohtake *et al.*, 1987; Wang *et al.*, 1990; Yamamoto *et al.*, 1993; Srinath *et al.*, 2002; Francisco *et al.*, 2002; Viti *et al.*, 2003). In a recent study, 16% of the Cr (VI) resistant bacterial strains isolated from tannery effluent had MIC more than 100 mg Cr (VI)/L (Srinath *et al.*, 2002). Another study reports that under *in vitro* conditions, Cr-tolerance may depend on the type of media used, the MIC obtained in the rich media are from two to five times higher than in minimal media because heavy metals can be complexed by some components of media, specially organic substances and phosphate (Mergeay, 1995). Bacterial sensitivity to metal toxicity is known to depend on their isolation site. In natural bacterial communities, the development of metal resistance is greatly enhanced by the horizontal dispersal of genetic information (Schmidt and Schlegel, 1989). Evolution of resistance via such transfer between natural bacterial isolates has

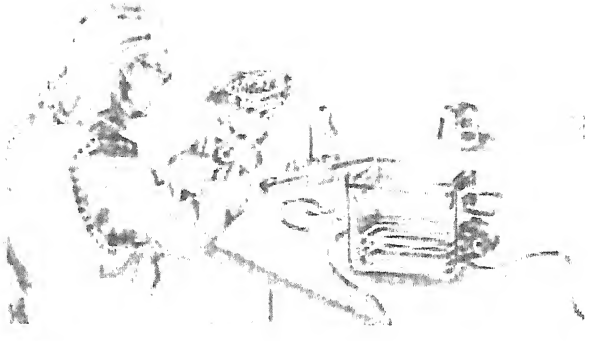
been shown to occur in situ and also under laboratory conditions (Top *et al.*, 1990). Widespread bacterial reduction of Cr (VI) to the less toxic Cr (III) ions is well known (Schmidt and Schlegel, 1989; Top *et al.*, 1990; Mergeay, 1995; Dhakephalkar *et al.*, 1996; Francisco *et al.*, 2002). In different bacteria, chromate reduction is either an aerobic or an anaerobic process (but not both) and is carried out either by soluble proteins or by cell membranes (Schmidt and Schlegel, 1989).

Several factors including mutagenic potential of chromium, a Cr (VI) reductional intermediate product for example Cr (III), a reaction to accumulated Cr or a selective stimulation of the control of binary fission, membrane proteins and lipids interaction and other intracellular mechanisms may play important role. Diverse mechanisms may be responsible for the development of resistance for Cr (VI) in microbes, including chromate efflux (Horitsu *et al.*, 1987).

Ingested Cr (VI) and Gut Microflora: The intestines have a huge population of bacteria (Table II.1) and the caecum harbors the largest number of bacteria as stated earlier (Simon and Gorbach, 1986; Guarner and Malagelada, 2003). Thus, bacteria may play an important role in protecting body from the toxicity of ingested chromium. The resident bacterial flora of the gastro-intestinal tract is exposed to Cr through ingestion of water and food contaminated with Cr. It has been reported that human ingestion of Cr (VI) in drinking water at levels of 1 to 10 ppm is safe due to high capacity of gastro-intestinal tract to reduce Cr (VI) to Cr (III). In long-term studies, rats are not adversely affected by 2.4 mg/kg/day of Cr (VI) as potassium dichromate in drinking water (Ivankovic and Preussmann, 1975).

A number of components in the intestines may be responsible for efficient handling of Cr (VI). It has been reported that bacteria isolated from faeces sequestrate 3.8 ± 1.7 µg Cr (VI)/10^9 bacteria thus the interesting derivation is that 11-24 mg Cr (VI) can be eliminated daily with faeces

(Olukoya *et al.*, 1997). Further, intestinal bacteria contain high amounts of GSH which efficiently reduces Cr (VI). Enterobacterial enzymes, such as nitroreductases can also reduce Cr (VI) (Owens and Hartman, 1986; Deflora and Wetterhahn, 1989). The findings indicate that the gut bacteria have marked capacity to cope with the increased load of chromium and may contribute in the protection against chromium toxicity up to certain extent. In addition, various antibiotic resistance shown by resident gut bacteria following chromium-ingestion also indicates that use of chromium as nutritional supplement / micronutrient may provide significant protection to the gut microflora, particularly *Lactobacillus*, against some of the commonly used antibiotics. The gut is the natural habitat for a large and dynamic bacterial community. Major functions of the gut microflora include metabolic activities that result in salvage of energy and absorbable nutrients, important trophic effects on intestinal epithelia and on immune structure and function, and protection of the colonized host against invasion by alien microbes (Srinath *et al.*, 2002).



Materials and Methods

METHODS AND MATERIALS

Animals: The study was carried out on Wistar rats weighing 200-250 g and Swiss mice weighing 20-25 g. The animals were bred and maintained in the Animal House of the Institute.

Chromium (VI): Potassium dichromate (hexavalent chromium; Cr (VI)) was of analytical grade and was purchased from M/s Qualigens, Lucknow. Chromium solution was prepared in drinking water and contained various concentrations of Cr(VI).

Preparation of stock solution of hexavalent chromium: Standard solution of 1000-ppm strength of chromium (VI) was prepared by dissolving $K_2Cr_2O_7$ in water.

Chromium (VI) treatment of Animals: The animals maintained on pellet diet were given *ad-lib* drinking water containing 10 ppm of Cr (VI) for different time periods.

Preparation of stock solution of hexavalent chromium: Standard solution of 1000-ppm strength of chromium (VI) was prepared by dissolving $K_2Cr_2O_7$ in water.

Chromium (VI) treatment of Rats: Wistar rats weighing 200-250 g and maintained on pellet diet in the animal house of this Institute were used. One group comprising of 6 rats were given *ad-lib* drinking water containing 10 ppm of Cr (VI) for 10-weeks. These rats were designated as Cr-stressed. The second group of 6 rats was given plain water.

Chromium (VI) treatment of Mice: Albino mice weighing 25-30 g and maintained on pellet diet in the animal house of this Institute were used. One group comprising of 6 mice were given *ad-lib* drinking water with 16mg/kg/d Cr (VI) for 3,6, and 9weeks.

Preparation of spleen cells: The animals were sacrificed by cervical dislocation and the spleens were removed aseptically. The spleen cells were teased out in cold MEM and a single cell suspension was obtained. The cells were washed and viable cell count done by trypan blue dye exclusion test (Chaturvedi *et al.*, 1978).

Preparation of splenic macrophages: Spleen cell suspension was incubated at 37 ^{0}C for 2h in an atmosphere of 5% CO_2 and the glass non-adherent cells were decanted gently. The glass-adherent cells were washed thrice with MEM and scrapped off with a policeman rod and resuspended as a single cell suspension. More than 95% cells were phagocytic (Chaturvedi *et al.*, 1982; 1983).

Separation of T and B lymphocytes: T and B lymphocytes were separated with glass wool and nylon wool columns by the technique of Julius et al. (1973) and Trigio & Cudkowicz (1974). The effluent (T lymphocytes) and the eluted cells (B lymphocytes) were centrifuged separately and resuspended in cold MEM and the purity was tested as described elsewhere (Tandon *et al.*, 1979).

Preparation of peritoneal macrophages: The peritoneal cavity of animal was lavaged with 5 ml. of heparinized Eagle's minimum essential medium containing non-essential amino acids (MEM). The aspirated cells were layered in glass Petridish and incubated at 37°C in presence of 5% CO_2 for 2h. The glass non-adherent cells were removed by washing three times. The glass-adherent cells scrapped off with a policeman rod. Cells were resuspended in MEM and a single cell suspension was prepared (Chaturvedi *et al.*, 1982; 1983).

Preparation of thymocytes: The single cell suspension of thymocytes was prepared in MEM with 10% FCS as described by Debetto *et al.* (1988). The viable cell count was done by trypan blue dye exclusion test.

Isolation of intestinal epithelial cells of various differentiation stages from Crypt to Villus: Over night fasted rats were sacrificed by cervical dislocation

and decapitation. Small intestine was removed and flushed gently with normal saline containing 1.0mM dithiothretol. Intestinal epithelial cells were prepared along crypt to villus axis on gradient of differentiation according to Weiser (1973). In brief, cecal end of the intestine was ligated and solution 'A' containing 1.5 mM KCl, 96 mM NaCl, 27mM sodium citrate, 8 mM KH_2PO_4 (pH 7.3) was filled after clamping the other end with artery forceps. The intestine was then immersed in solution 'A' and incubated at 37°C for 15 min in a constant water bath. After incubation the intestine was emptied and fluid discarded. The intestine was now filled with solution 'B' containing 1.5mM EDTA and 5 mM dithiothretol in PBS (pH 7.2) and immersed in solution 'B' for incubation. After 4 min incubation, the contents were emptied into a plastic centrifuge tube to recover the first epithelial cell population. The intestine was filled with solution 'B' for different time intervals and the process was repeated for several times to collect cell population of differentiation stages. Cell population were centrifuged at 900 g for 5 min and washed twice with 4 mM EDTA solution containing 15 mM β-mercaptoethanol (pH 7.4) to remove phosphate buffer. For this experiment, epithelial cells in their sequence of dissociation from the intestine were pooled into three fractions on the basis of their protein content and alkaline phosphatase activity as described by Panini *et al.* (1979). These fractions were designated as Upper Villi, Middle Villi and Crypt respectively. From each fraction $5x10^6$ cells/ml were used in the tests.

Experiments on intestinal cells *in situ*: Laparotomy on each rat was performed by midline incision under light ether anaesthesia. The intestine was washed with normal saline, using a syringe and a blunt needle, through two small cuts. One was made slightly distal to the duodeno-jejunal junction and another at the distal end of ileum. After washing, the opening was ligated and 10 cm length loops were prepared from the upper end of intestine using sterile threads (Rastogi *et al.*, 1988). Test solution was administered into the loops through proximal opening, which was then immediately ligated. Control loops

contained normal physiologic saline solution. The whole intestine was kept *in situ* and the abdomen stitched immediately. Proper breathing and anaesthesia of the animal was maintained throughout the experiment. Loops were removed after 30 min incubation time, gently blotted on filter paper, and the contents were drained into graduated tubes. The luminal fluid was made up to desired volume and centrifuged at 500 *g* for 5 min to remove any intestinal debris.

Estimation of chromium (VI) reduction by the cells: Potassium dichromate (hexavalent chromium; Cr (VI)) was of analytical grade and was purchased from M/S Qualigens, Lucknow. Chromium solution was prepared in MEM containing 10 or 25 μg/ml of chromium. $5x10^6$ cells were suspended in one-ml chromium solution and incubated at 37°C. At different time periods an aliquot was taken, the cells were removed by spinning and the reduction of Cr (VI) was assayed in the cell-free supernatant spectrophotometrically at 540nm using diphenylcarbazide reagent (Urone, 1955). The experiments were setup in triplicate and were repeated thrice. The mean data ± SD has been presented as percentage reduction of Cr (VI).

Isolation and Identification of resident gut microflora: At the end of 10th week all the animals were sacrificed. The abdomen was opened with full aseptic precautions. The caecum was located and injected with 5.0 ml of sterilized phosphate buffered saline (PBS) from one end. After 2 min. a small nick was made on the caecum and the fluid was collect in a sterilized Petridish. One loopful of the caecal fluid was streaked out on nutrient agar plate. The caecal fluid from the Cr-stressed rats was streaked on nutrient agar plates containing 10 mg/L concentration of Cr (VI). After 24 h incubation the morphology of different bacterial colonies was recorded and the smears were stained with Gram's stain. The bacteria were identified on the basis of biochemical reactions according to the Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1989). The bacteria selected for further study from

the two groups of rats were *Escherichia coli*, *Pseudomonas* sp. and *Lactobacillus* sp.

Growth pattern of normal and Cr-stressed intestinal microflora in presence of Cr (VI): Growth curves of bacterial isolates were prepared as described. A single colony of each isolated bacteria was subcultured in 5.0 ml. of peptone water. Growth phase of the bacteria were obtained and about $5x10^7$ bacterial cells were suspended in 100 ml. of peptone water containing various concentrations of Cr (VI). One control was also included for each bacterium without containing Cr (VI). The Optical Density of an aliquot of the culture was measured at 610 nm at 0, 2, 4, 6, 8,10, 12, 24 and 30 hrs after incubation. The tests were setup in triplicate and the mean value from all the animals of the group have been presented. The concentration of viable cells was determined by plating 100 µl of appropriately diluted culture on to nutrient agar and incubating the plates at 35°C for 24 h.

Curing of Plasmid: Plasmids of all the strains were cured by treatment of the bacterial cultures with acridine orange (125 µg/ml) at 42°C for 24 h as described else where (Dhakephalkar *et al.*, 1996).

Estimation of chromium (VI) reduction by the bacteria: Potassium dichromate (Cr (VI)) was of analytical grade and was purchased from M/s Qualigens, India. Chromium solutions were prepared in Peptone water containing 10, 25, 50 or 100 mg/L of Cr (VI). Growth phase of the bacteria were obtained and about $5x10^7$ bacterial cells were suspended in 100ml Peptone water with or without various concentrations of Cr (VI). They were incubated at 37°C on a shaker at 100 rpm. At different time periods an aliquot was taken and the cells were removed by spinning at 8000g for 10 min. The reduction of Cr (VI) was assayed in the cell-free supernatant spectrophotometrically at 540 nm using diphenylcarbazide reagent (Urone,

1955). The experiments were setup in triplicate. The mean data±SD has been presented as percentage reduction of Cr (VI).

Determination of minimal inhibitory concentration of Cr (VI): The minimal inhibitory concentration (MIC) of chromium at which no colony growth occurred was determined by broth agar dilution method (Luli *et al.*, 1983). The bacteria were inoculated into 25 ml peptone water (HiMedia, India) consisting of 1.0% (w/v) peptone and 5.0% (w/v) NaCl in conical flasks and incubated at 28°C at 150 rpm to achieve log phase cultures. Nutrient agar plates containing different concentrations (50 to 200 mg/L) of Cr (VI) were inoculated from the exponential growing cultures of each bacterial strain. These plates were incubated at 37°C for 48 h. The lowest concentration of Cr (VI) at which no bacterial growth occurred was considered as MIC.

Determination of antibiotic sensitivity: All the strains of bacteria isolated from the two groups of rats were tested for antibiotic sensitivity following the National Committee for Clinical Laboratory Standard (NCCL) disc diffusion method. Sensitivity of different cured strains to various antibiotics was also tested. The following antibiotic discs were used: Tetracycline (30 μg), Cotrimazol (25 μg), Trimethophorin (5 μg), Chloramphenicol (30 μg), Streptomycin (30 μg), Gentamycin (10 μg), Nalidixic acid (30 μg), Ampicillin (50 μg) and Kanamycin (30 μg).

Statistical analysis: Student's t-test was used for statistical evaluation of the data. A p value of less than 0.05 was considered significant.

Dengue Virus: Dengue type 2 virus (DV), strain P23085 was used in the form of infected infant mouse brain suspension (Chaturvedi *et al.*, 1978). Mice were inoculated with 1000 LD 50 of DV intracerebrally (ic) in doses of 0.03 ml.

Collection of blood sample: Animals were sacrificed and 1 ml. of blood sample were collected from the jugular vein, in tubes containing 0.5 ml. of 10%

K_3 EDTA solution as, anticoagulant (freshly prepared). The samples were shaken gently.

Haematological study: The following estimations were performed immediately with the help of a fully automatic haematology analyzer (Bayer; Technicon H1*E): total leucocyte count (TLC), differential leucocyte count (DLC), total RBC count, haematocrit (Hct), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and platelet Counts (Plt). Standard chemicals and reagents supplied by the same company were used.

Weight of spleen: At the time of killing the body weight of mouse was recorded. The spleen was removed. The weight of the spleen was represented as mg/100g body weight of the mouse.

Preparation of spleen cell culture: The spleen cells were teased out in cold MEM and a single cell suspension was prepared. The viable nucleated cell count as ascertained by trypan blue dye exclusion test was more than 95%.

Spleen cell proliferation assay: The cell suspension of spleen was adjusted to 2x 10 6 cells/ml in Eagle's MEM media supplemented with 10% FCS. Cell cultures were setup in 96 wells U- bottom microtitre plate in triplicate. Each well contained 0.2ml cell suspension with or without 5 μg/ml. Concanavalin (Con) A. The cultures were incubated for 72hrs at 37°C in presence of 5% CO_2. Five hours before the termination of the cultures, 1 μCi of [^{3}H]-Thymidine was added to each well. Cells were harvested and washed repeatedly with PBS (pH 7.2). Cell associated radioactivity was measured in Scintillation vials containing 5 ml. Scintillation fluid and were counted in Beta counter (Liquid Scintillation Fluid Analyser, TRI CARB 2900TR, Packard Biosciences Company). The data has been presented as mean value $\pm$ SD of counts per minute (cpm). The stimulation Index was calculated as ratio of the CPM to Con A treated cultures to that of media alone.

Preparation of spleen homogenate: Spleens were washed free of blood, homogenized in a tissue homogenizer giving a 10% solution (w/v) in chilled phosphate buffered saline (pH 7.2). The homogenate was centrifuged in the cold at 3000 rpm for 10 min. and clear supernatant was stored in small aliquots at –20°C. Normal mouse spleen homogenates were prepared similarly and used as control.

Cytotoxicity test: The cytotoxic activity of the spleen homogenate was tested using normal mouse spleen cells as target. The tests were carried out in microtitre plates with 96 wells, using 0.1 ml. Volume of each preparation. The target single cell suspension of normal mouse spleen was prepared as described before. 0.1 ml. Volume of cell suspension containing 2x 10^6 cell/ml was added in each well. After thorough mixing, the trays were kept at 4°C in a refrigerator for one hour. The trays were rocked every 10 min during the period of incubation. Then the cell viability was measured by MTT {3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium bromide} assay (Mosmann, 1983). Throughout the test all procedures were carried out at 4°C in ice bath.

MTT assay: After 1 hour incubation 20 µl of MTT solution (5 mg/ml) was added to each well and the plates were incubated at 37°C for 4 hours. After 4 hours incubation media was aspirated carefully with the help of syringe and 200 µl of DMSO was added to each well, and mixed thoroughly to dissolve the dark blue crystal. After waiting for a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on Micro ELISA Reader using a test wavelength 570 nm. The MTT assay was done in triplicate and the mean value of % of non-viable cell ± as obtained in repeated experiments are presented.

Phagocytic activity of splenic macrophages: Spleen cell suspension layered on glass slides placed in Petridishes was incubated at 37°C for 2h in an atmosphere of 5% CO_2. The glass-non-adherent cells were decanted gently. The

Results and Discussion

EXPERIMENT 1. EFFECTS OF HEXAVALENT CHROMIUM ON BLOOD CELLS DURING DENGUE VIRUS INFECTION

INTRODUCTION

Chromium enters the body through the lungs, gastro-intestinal tract, and to a lower extent through skin (Hamilton and Wetterhahn, 1988). Non-occupational exposure occurs via ingestion of chromium containing food and water (Langard 1982; Pedersen, 1982). Regardless of route of exposure Cr(III) is poorly absorbed whereas Cr(VI) is more readily absorbed (Hamilton and Wetterhahn, 1988). Further, absorption of Cr(VI) is poorer by oral route therefore, it is not very toxic when introduced by oral route (Deflora, 1997). Most of the chromium absorbed by inhalation exposure, in comparison to oral administration, is distributed in the lungs, liver, kidneys, RBC, plasma, spleen, bone-marrow (Langard, 1982). All the ingested Cr(VI) is reduced to Cr(III) before entering in the blood stream (Kerger *et al.* 1996). The main routes for the excretion of chromium are via kidney/urine and the bile/feces (Guthrie 1982, Langard 1982).

During dengue virus infection various changes occur in the peripheral blood cells. The total leucocyte count in dengue patients varies from mild leucopenia to moderate leucocytosis with predominance of lymphocytes. The haematocrit value increases during the first 3 days but gradually decreases from 4 to 9 day. Thrombocytopenia is one of the simple diagnostic criteria proposed by WHO for clinical diagnosis of DHF. The platelet count is usually normal during the first three days. Thrombocytopenia in more than 80% cases begins during febrile stage and reaches its lowest value during the shock phase of illness. The present experiment was therefore, conducted to study the effect of chromium (VI) on peripheral blood cells during dengue virus infection of mice.

METHODS AND MATERIALS

Animals: The study was carried out on Swiss mice weighing 25-30 gm.

Chromium treatment of mice: Each group comprising of 6 mice were given ad lib drinking water containing chromium (VI) for 3,6 and 9 weeks.

Dengue Virus: Dengue type 2 virus (DV), strain P23085 was used in the form of infected infant mouse brain suspension (Chaturvedi *et al.*, 1977). Mice were inoculated with 1000 LD_{50} of DV intracerebrally (ic) in doses of 0.03 ml.

Collection of blood sample: Animals were sacrificed and 1 ml. of blood sample were collected from the jugular vein, in tubes containing 0.5 ml of 10% K3 EDTA solution as, anticoagulant (freshly prepared). The samples were shaken gently.

Haematological study: The following estimations were performed immediately with the help of a fully automatic haematology analyzer (Bayer; Technicon H1*E): total leucocyte count (TLC), differential leucocyte count (DLC), total RBC count, haematocrit (Hct), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC) and platelet Counts (Plt).

Plan of study: Mice maintained on pellet diet in the animal house of this Institute were used. One group of mice was given *ad-lib* drinking water with 16 mg/kg/d of Cr (VI) and the second group was given plain water to drink. At the 3, 6 and 9 week of Cr (VI) drinking, a group of mice were inoculated *i.c.* with DV in doses of 1000 LD_{50}. The mice were killed in groups of 6 at the 4^{th} and 8^{th} day of the virus inoculation and various investigations were done. The plan has been summarized in the Figure IV.1.1.

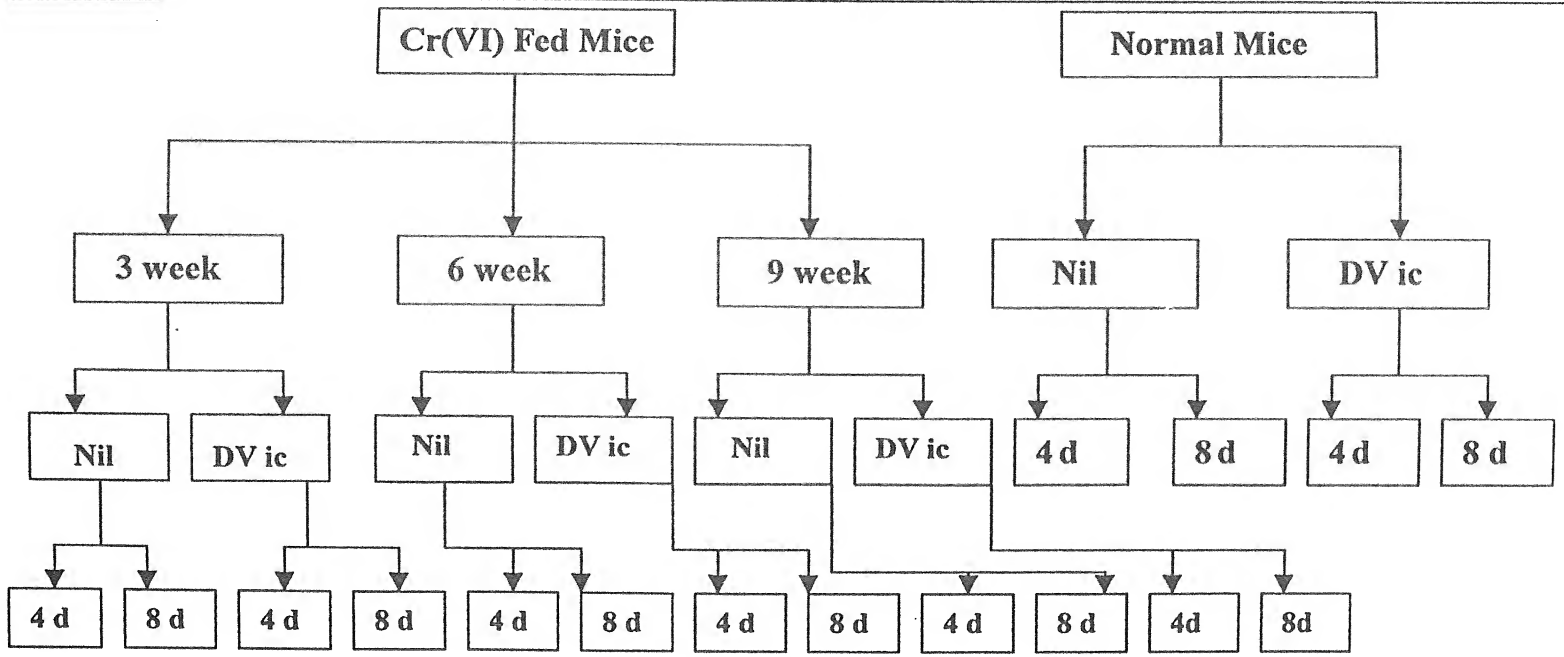


Figure IV.4.1: Schematic presentation of the plan of study. DV ic, mice inoculated with dengue virus intracerebrally; Nil, mice not given dengue virus; 4d, mice killed on the 4th day of the virus inoculation; 8d, mice killed on the 8th day of the virus inoculation; mice not given virus were killed also killed on the similar days.

RESULTS

Effects of dengue virus inoculation on mice: The mice remained apparently healthy up to the 4th day. The back was arched and the fur was ruffled from the 5th day. By the 8th day the mice became seriously ill with the development of hind limb paralysis (Figure IV.1.2 and 3) and all of them died by the 10th day. Therefore the experiments were conducted on the 4th and the 8th day post-inoculation of the dengue virus.

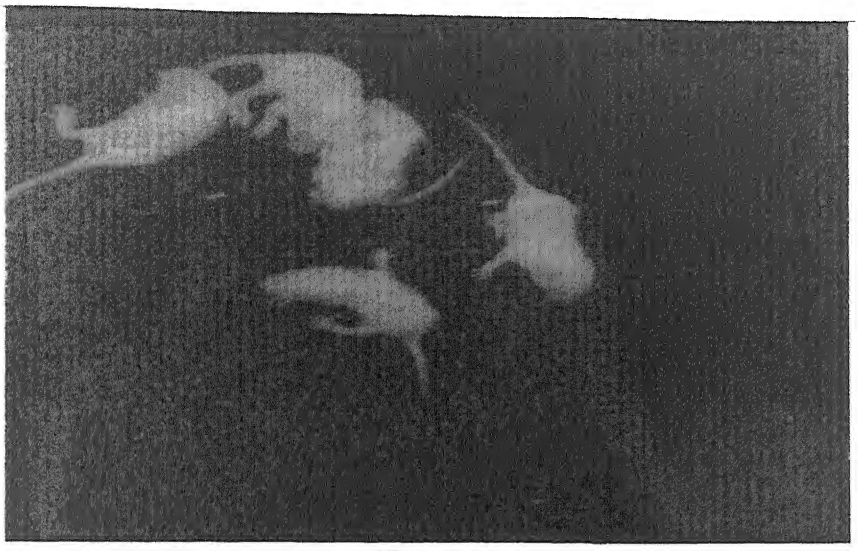
Effects on leucocytes

The data presented in Table IV.1.1 shows that in normal control mice the total leucocyte count was $7\pm0.1x10^3$/Cu mm with 83% lymphocytes and 14% granulocytes. By giving Cr(VI) in drinking water for 3 weeks the total count was reduced to $5\pm1x10^3$/Cu mm, with 21±8% granulocytes. At the 6th and 9th week there was not much difference in total leucocyte counts but the percentage of granulocytes and monocytes was increased with consequent reduction in lymphocytes (Table IV.1.1).

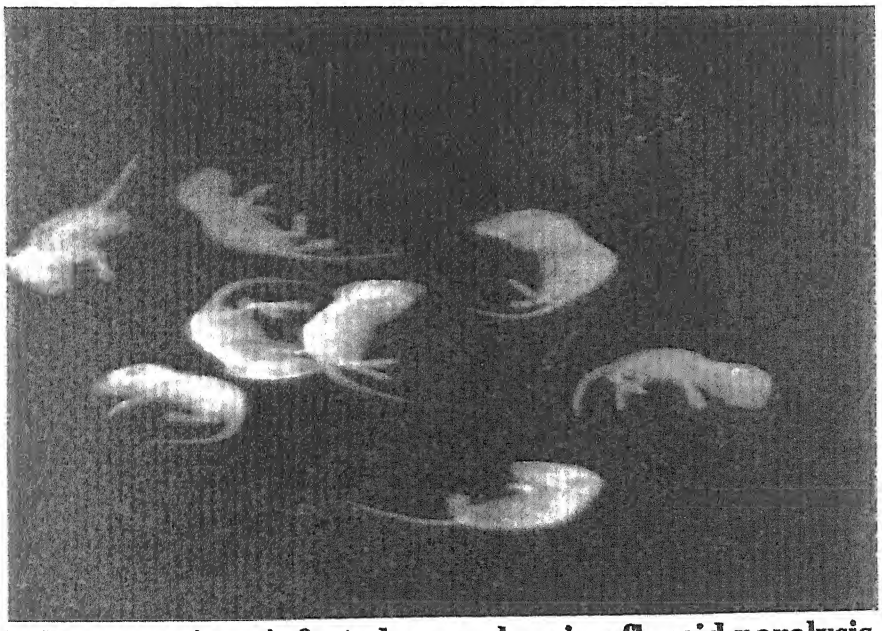
Table : IV.1.1 Leucocytes count in Chromium(VI) Treated Mice

Group*	Total Leucocyte Count /Cmm	Differential leucocyte count (Percent)		
		Lymphocyte	Monocytes	Granulocytes
Control	7±0.1	83±1	3±0.1	14±0
Cr(VI) 3 W	5±1	70±8	9±1	21±8
Cr(VI) 6 W	7±1	66±6	11±2	23±5
Cr(VI) 9 W	6±1	50±6	15± 3	35±7

***Control, normal mice; Cr(VI) 3W, mice fed chromium for 3 weeks; Cr(VI) 6W, mice fed chromium for 6 weeks; Cr(VI) 9W, mice fed chromium for 9 weeks**



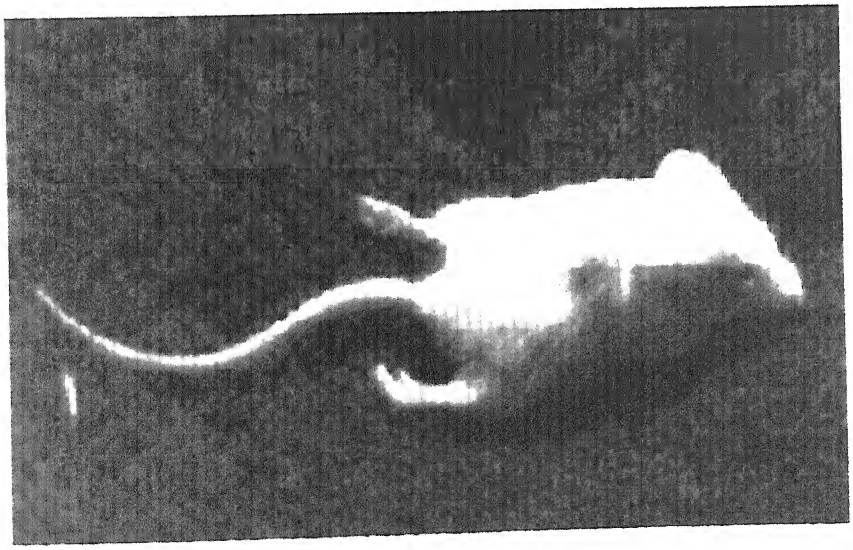
a. Healthy pups



b. Dengue virus infected pups showing flaccid paralysis.

Figure IV.1.2 Clinical picture of Dengue virus in 5 day old suckling mice

a. Healthy normal control mice



b. Dengue virus infected adult mice showing flaccid paralysis.

Fig. IV.1.3: Clinical picture of dengue virus infected adult mice

When the normal mice were inoculated intracerebrally with 1000 LD_{50} of the dengue virus, a sharp reduction in the total leucocyte count was seeing at the day 4 when the mean value was $5\pm2x10^3$/Cu mm. On the 8th day there was little improvement in the counts. The predominant cells in the blood remained lymphocytes in all the groups (Table IV.1.2).

Table IV.1.2: Peripheral blood leucocytes count in DV-infected mice

Group*	Total Leucocyte Count /Cmm	Differential leucocyte count (Percent)		
		Lymphocyte	Monocytes	Granulocytes
Control	7±0.1	83±1	3±0.1	14±0
DV D4	3±2	87±5	2±0.5	11±5
DV D8	5±1	80±3	3±1	18±2

***Control, normal mice; DV, dengue virus; D4, 4th day after infection with DV; D8, 8th day after infection with DV**

The data summarized in Table IV.1.3 show the effect of drinking of Cr(VI) on the leucocyte counts during dengue virus infection. The finding show a reduction in total leucocyte count at 3 week and 9 week of Cr(VI) drinking but no significant difference was seen at the 6th week. The most significant finding was a marked increase in the percentage of granulocytes at all the periods of Cr(VI) drinking with the consequent decrease in the lymphocytes (Table IV.1.3).

Table : IV.1.3 Leucocytes count in Chromium(VI) treated mice after DV infection

Group*	Total Leucocyte Count /Cmm	Differential leucocyte count (Percent)		
		Lymphocyte	Monocytes	Granulocytes
Control	7±0.1	83±1	3±0.1	14±0
Cr(VI) 3 W				
DV D4	4±2	45±3	7±1	48±3
DV D8	5±1	42±5	8±2	50±5
Cr(VI) 6 W				
DV D4	6±1	50±3	8±3	42±3
DV D8	6±1	47±5	8±3	45±5
Cr(VI) 9 W				
DV D4	3±1	30±12	10±1	60±15
DV D8	4±1	40±6	8±2	52±6

***Control, normal mice; DV, dengue virus; D4, 4th day after infection with DV; D8, 8th day after infection with DV; *Control, normal mice; Cr(VI) 3W, mice fed chromium for 3 weeks; Cr(VI) 6W, mice fed chromium for 6 weeks; Cr(VI) 9W, mice fed chromium for 9 weeks;**

Effects on Red Blood Cells

The total red blood cell count in normal mice was $8\pm0.3x10^6$/Cu mm. By feeding Cr(VI) the red blood cell count increased to $11\pm1x10^6$/Cu mm (Table IV.1.4). This was associated with a reduction in the mean corpuscular volume and mean corpuscular haemoglobin. At the 6th and 9th week there was not much change in total red blood cell count but the haematocrit and the haemoglobin concentration was reduced especially at the 9th week.

Table: IV.1.4 Red Blood Cell Indices of Chromium(VI) Treated Mice

Group*	RBC	MCV	HCt	MCH	MCHC	RDW	Hb
Control	8±0.3	46±0.3	38±0.1	14±0.1	30±0.40	8±1	12±0.10
Cr(VI) 3 W	11±1	39±2	40±3.6	12±2	30±1.4	9±0.3	12.0±0.7
Cr(VI) 6 W	8±0.2	50±2.36	39±3	12±1	29±2	9±0.05	11±0.2
Cr(VI) 9 W	7±0.5	37±1	26±2	13±0.43	35±1.0	7±0.1	9.0±0.7

***Control, normal mice; Cr(VI) 3W, mice fed chromium for 3 weeks; Cr(VI) 6W, mice fed chromium for 6 weeks; Cr(VI) 9W, mice fed chromium for 9 weeks; RBC,Red blood cell ; MCV, Mean corpuscular volume; HCt, Haematocrit; MCH, Mean corpuscular haemoglobin; MCHC, Mean corpuscular haemoglobin concentration; RDW, Red blood cell distribution width; Hb, Haemoglobin**

The data summarized in Table IV.1.5 shows the red blood cell indices in normal mice inoculated with the dengue virus. The findings show a significant decrease in the total red blood cell counts, haematocrit value and haemoglobin concentration at both, the 4^{th} and 8^{th} day of dengue virus inoculation.

Table :- IV.1.5 Red blood cell indices of DV infected mice

Group*	RBC	MCV	HCt	MCH	MCHC	RDW	Hb
Control	8±0.3	46±0.3	38±0.1	14±0.1	30±0.40	8±1	12±0.10
DV D4	5±0.5	44±0.8	22±2	13±0.47	31±1.38	7±0.2	7±0.60
DV D8	6±0.03	44±0.50	24±0.20	13±0.24	31±0.63	7±0.01	7±0.00

***Control, normal mice; DV, dengue virus; D4, 4^{th} day after infection with DV; D8, 8^{th} day after infection with DV; RBC,Red blood cell ; MCV, Mean corpuscular volume; HCt, Haematocrit; MCH, Mean corpuscular haemoglobin; MCHC, Mean corpuscular haemoglobin concentration; RDW, Red blood cell distribution width; Hb, Haemoglobin**

When Cr(VI) fed mice were inoculated with dengue virus the total red blood cell count remained similar to that in controls. The significant change

was reduction in the haematocrit values and haemoglobin concentrations, specially at the 9th week of Cr(VI) drinking (Table IV.1.6).

Table IV.1.6: Red Blood Cell Indices of Chromium(VI) Treated Mice After DV Infection

Group*	RBC	MCV	HCt	MCH	MCHC	RDW	Hb
Control	8±0.3	46±0.3	38±0.1	14±0.1	30±0.40	8±1	12±0.10
Cr(VI) 3 W							
DV D4	9±1	40±0.7	35±4.5	13±0.2	32±0.5	8±0.1	11±1.3
DV D8	11±1.4	40±4.2	45±9	12±3	29±5.37	8± 0.1	13± 3
Cr(VI) 6 W							
DV D4	8±1	50±3	40±1	12±2	29±2	7±1	11±0.5
DV D8	7±0.5	53±3	39±1.4	13±1	28±1	7±3	11±0.7
Cr(VI) 9 W							
DV D4	8±0.8	36±0.9	28±3	12±0.3	34±0.5	6±2	9±0.9
DV D8	6±0.6	35±1	22±2	13±1	37±4	7±3	8±0.2

***Control, normal mice; DV, dengue virus; D4, 4th day after infection with DV; D8, 8th day after infection with DV; *Control, normal mice; Cr(VI) 3W, mice fed chromium for 3 weeks; Cr(VI) 6W, mice fed chromium for 6 weeks; Cr(VI) 9W, mice fed chromium for 9 weeks; RBC, Red blood cell ; MCV, Mean corpuscular volume; HCt, Haematocrit; MCH, Mean corpuscular haemoglobin; MCHC, Mean corpuscular haemoglobin concentration; RDW, Red blood cell distribution width; Hb, Haemoglobin**

Effects on Platelets

The platelet count in the blood of normal control mice was 374±34x10^6/Cu mm. The findings presented in Figure IV.1.4 show that by drinking Cr(VI) the total platelet count was significantly increased being 657±289 x10^6/Cu mm at the 3 week to 736±185 x10^6/Cu mm at the 9th week. When normal control mice was inoculated with the dengue virus a significant reduction in the platelet count was seeing both at the 4th and 8th day being 191±162 and 222±106x10^6/Cu mm respectively. When Cr(VI) treated mice

were inoculated with dengue virus, the virus-induced reduction in platelet count was not observed at all the periods as the counts remained 370±139 to 626±134 x10^6/Cu mm (Figure IV.1.4).

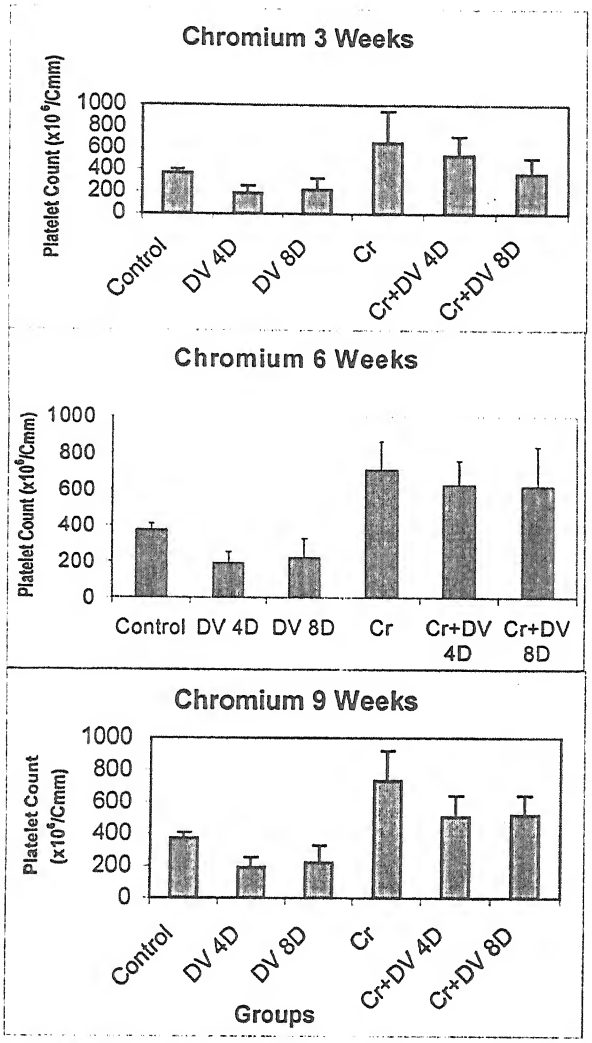


Figure IV.1.4: Effects of subtoxic dose of Cr (VI) on platelets during dengue virus infection. Cr, chromium; DV, dengue virus; 4,8, days after the virus infection

The finding presented in Table IV.1.7 shows the mean platelet volume and platelet distribution width. It was observed that by drinking Cr(VI) the

mean platelet volume was slightly reduced. A greater change was observed in platelet distribution width by drinking Cr(VI).

Table :-IV.1.7 Platelet Count of Chromium(VI) Treated Mice

Group*	Plt (10^6/Cmm)	MPV	PDW
Control	374±4.0	7.00±0.5	9.05±0.05
Cr(VI) 3 W	657±289	5.28±0.24	5.95±0.58
Cr(VI) 6 W	711±154	6.00±0.5	7.34±0.7
Cr(VI) 9 W	736±185	6.26±0.24	8.02±0.71

***Control, normal mice; Cr(VI) 3W, mice fed chromium for 3 weeks; Cr(VI) 6W, mice fed chromium for 6 weeks; Cr(VI) 9W, mice fed chromium for 9 weeks; Plt, Platelet count; MPV, Mean platelet volume; PDW, Platelet distribution width**

When normal mice was inoculated with dengue virus a marked reduction in the mean platelet volume was observed at the 8th day but no change was observed in platelet distribution width (Table IV.1.8).

Table IV.1.8: Platelet count of DV infected mice

Group*	Plt (10^6/Cmm)	MPV	PDW
Control	374±4.0	7.00±0.5	9.05±0.05
DV D4	191±62	6.7±0.17	8.54±0.23
DV D8	222±106	5.9±0.2	8.8±0.1

***Control, normal mice; DV, dengue virus; D4, 4th day after infection with DV; D8, 8th day after infection with DV; Plt, Platelet count; MPV, Mean platelet volume; PDW, Platelet distribution width**

Inoculation of dengue virus in Cr(VI) treated mice resulted in the decrease of mean platelet volume and platelet distribution width especially at 3 weeks (Table IV.1.9).

Table IV.1.9: Platelet Count in Chromium(VI) Treated Mice during DV Infection

Group*	Plt (10^6/Cmm)	MPV	PDW
Control	374±4.0	7.00±0.5	9.05±0.05
Cr(VI) 3W			
DV D4	540±173	5.32±0.21	6.20±0.45
DV D8	370±339	5.58±0.52	6.78±1.70
Cr(VI) 6 W			
DV D4	626±134	6.08±1.2	7.12±0.80
DV D8	617±219	7.00±0.5	7.45±2
Cr(VI) 9 W			
DV D4	510±130	6.40±0.28	8.08±1.7
DV D8	521±119	6.45±0.55	7.15±1.05

***Control, normal mice; DV, dengue virus; D4, 4th day after infection with DV; D8, 8th day after infection with DV; *Control, normal mice; Cr(VI) 3W, mice fed chromium for 3 weeks; Cr(VI) 6W, mice fed chromium for 6 weeks; Cr(VI) 9W, mice fed chromium for 9 weeks; Plt, Platelet count; MPV, Mean platlet volume; PDW, Platelet distribution width**

DISCUSSION

The most significant finding of the present study the marked reduction in lymphocyte percentage and increase in the granulocyte, monocyte and platelet counts in mice fed Cr(VI) with drinking water. Differential leucocyte counts revealed that percentage of lymphocytes decreased by 50%. The absolute lymphocyte number was decreased from 6000 cells/mm^3 to 3500cells/mm^3. Similar findings have been reported in fishes exposed to chromium by Khangarot *et al.* (1999) and Arunkumar *et al.* (2000).

It was also observed that percentage of polymorphonuclear and monocytes was increased gradually from 3 to 9 weeks. Number of monocytes

increased from 210 cells/mm^3 to 900 cells/mm^3 whereas polymorphonuclear increased from 980 cells/mm^3 to 2100 cells/mm^3 at the 9^{th} week of Cr(VI) drinking. Goldman and Karotin (1955) have reported a significant increase in polymorphonuclear cells when a 25 years old woman drank a solution containing potassium dichromate. Routine hematological examinations revealed no change in rats exposed to 3.6 mg Cr(VI)/kg./day as potassium chromate in the drinking water for 1 year (Mackenzie *et al.*,1958). On the other hand, rats exposed to atmosphere containing soluble potassium chromate had significantly increased levels of polymorphonuclear and monocytes (Cohen *et al.*,1998). Khangarot observed that fishes exposed to different concentration of Cr(VI) had significantly increased polymorphonuclear and monocytes.

The normal mice showed a 48% decrease in total leucocyte count at the 4^{th} day of DV inoculation. The number of polymorphonuclear was also reduced. Pimpan and Prasert (1993) have also reported that white blood cell count in dengue fever patients varies from mild leucopenia to mild leucocytosis. During leucopenia there is usually a fairly normal differential count of leucocytes and lymphocytes, the polymorphonuclear concentration is depressed between 3^{rd} to 8^{th} day (Pimpan and Prasert, 1993). At the 8^{th} day of post inoculation of DV number of lymphocytes was also decreased, when absolute count was considered this decrease in number of lymphocytes was 6000 cells/mm^3 to 3200 cells/mm^3, about 47%. Halstead *et al.* (1969) reported that at the initial stage of dengue fever there is a progressive shift of neutrophils to the left that is, an increase of immature non-segmented nuclear (band), which persists into convalscent phase, and there is a marked depletion of circulating lymphocytes.

When Cr(VI) fed mice were inoculated with DV it was found that total leucocyte count was decreased more or less similar to mice inoculated only with DV, but most interesting finding was marked reduction in number of lymphocytes that means Cr(VI) drinking during DV infection reduces the large

number of lymphocytes, on the other hand number of polymorphonuclears were not affected very much by DV inoculation in Cr(VI) fed mice in comparison to the DV inoculated mice.

By drinking Cr(VI) for 3 weeks the red blood cell count was increased by 29% while at 6^{th} and 9^{th} weeks it remains similar. Glaser *et al.* (1988) reported that rats exposed to 0.1mg Cr/m3 as a 3:2 mixture of Cr(VI) trioxide and Cr(III) trioxide for 18 months have increased red blood cell counts .

A marked reduction in mean corpuscular volume and mean corpuscular haemoglobin was observed at the end of 3^{rd} week of Cr(VI) drinking. The MCV was reduced by 18% and MCH reduced by 15%. Similar findings have been reported in a number of studies. Rats and mice fed potassium dichromate showed slightly reduced MCV and MCH values at earlier periods and low doses of Cr (VI) but the decreased was marked with the higher doses given for longer periods (NTP 1996a, 1996b, 1997). In the present study it was observed that after 9 weeks of drinking Cr(VI) haematocrit and haemoglobin contents were decreased. The haematocrit value was decreased by 32% whereas haemoglobin was decreased by 23%. The mechanism of decrease of haemoglobin appears to be due to inhibition of its biosynthesis. In the initial stage of pathway, succinyl Co-A combines with glycine to form the first of the intermediates, δ amino levulinic acid. Chromium may cause an increase in SDH synthesis and therefore decrease the succinyl Co-A pool. It may also interact with serine, which is the precursor of glycine, and therefore decrease the serine pool. Chromium causes the DNA damage, producing inhibition of the activities of one or more enzymes involved in heme synthesis (Moore *et al.* 1980, Soria *et al.* 1995). Sharma *et al.* (1978) reported that in a 18 years old woman who ingested few grams of $K_2Cr_2O_7$, decreased haemoglobin and haematocrit were found 4 day after ingestion. In another study, a 44 year old man had decreased haemoglobin levels 9 day after ingestion of chromium (Saryan and Reedy, 1988). Khangarot *et al.* (1999) observed dose dependent

decrease in haemoglobin content in the catfish exposed to chromium for 28 days in a static bioassay test procedure.

Haemoglobin concentration was decreased by 42% at the 8th day in normal mice inoculated with DV. The haematocrit value was also decreased by 40%. Pimpan and Prasert (1993) studied that during DHF haematocrit value increases initially but later on it was decreases from 4th to 8th day. Gastrointestinal bleeding may initially be occult and usually manifest as a drop in haematocrit without clinical improvement (Kuberski *et al.* 1977, Fagbami *et al.* 1995).

When Cr(VI) was given to mice the platelet counts were increased gradually from 3 to 9 weeks, the increase being 90% at the 9th week. Khangarot *et al.* (1999) have reported a significant increase in platelets in fishes exposed to hexavalent chromium. In the present study when normal mice was inoculated with DV platelet count was decreased by 50% and 40% at the 4th and 8th day post inoculation of DV respectively. Thrombocytopenia is one of the simple diagnostic criteria proposed by WHO for the clinical diagnosis of DHF (Pimpan and Prasert, 1993). During DHF platelet count is usually normal during the first three days .The number of the platelet decreases from 4th to 9th day and returns to normal with in seven to ten days after the beginning of the disease. The cause of thrombocytopenia is either impaired megakaryocytes production or increased platelet destruction (Nelson *et al.*, 1964; Halstead *et al.*, 1969). When Cr(VI) treated mice was inoculated with DV the platelet count decrease at 4th and 8th day reduced by lower extent in comparison to DV alone group. That means drinking of Cr(VI) prevents the platelet count fall during DV infection. A comparison of the effect of Cr(VI) and DV on blood cells is presented in Fig. IV.1.5.

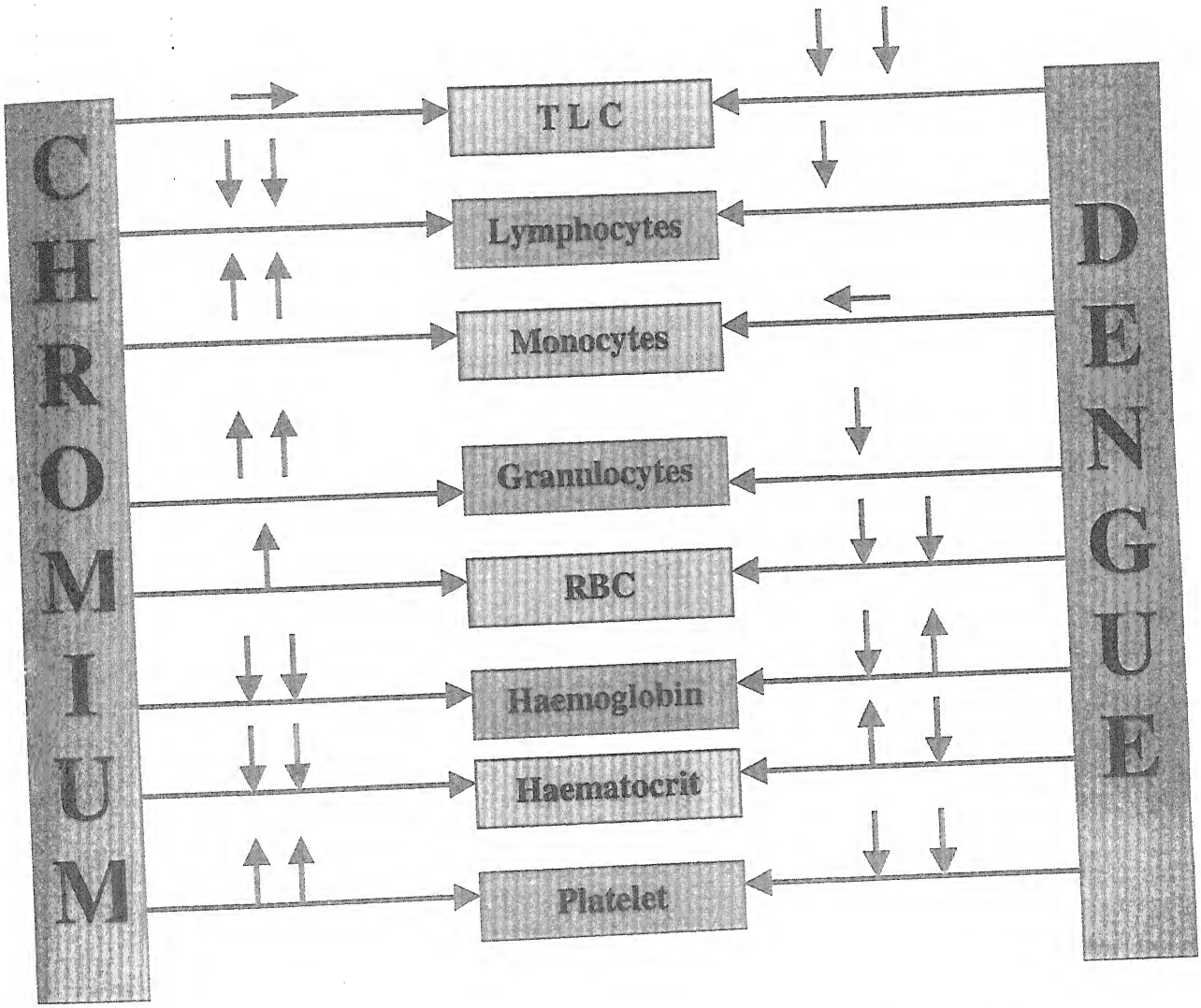


Fig. IV.1.5: Comparison of the effects of DV and Cr (VI) on blood cells.

EXPERIMENT 2. EFFECTS OF HEXAVALENT CHROMIUM ON SPLEEN DURING DENGUE VIRUS INFECTION

Introduction

Spleen is one of the larger lympho-reticular organ that plays important role in the defence mechanisms of body. It has a variety of immunologically reactive cells for example lymphocytes, macrophages etc. Any foreign substance that enters the blood circulation reaches spleen. It was therefore, considered important to study effects of Cr (VI) on the spleen and its cells during dengue virus infection.

METHODS AND MATERIALS

Animals: The study was carried out on Swiss mice weighing 25-30 gm.

Virus: Dengue type 2 virus (DV) P23085 was used in the form of infected infant mouse brain suspension (Chaturvedi *et al.*, 1978). Mice were inoculated with 1000 LD 50 of DV *i.c.* in doses of 0.03 ml. And were killed in batches of 6.

Plan of study: The animal groups and the plan of study was just the same as described in Experiment 1 (Figure IV.1.1). At the time of killing the body weight of mouse was recorded and the spleen was removed with full aseptic precautions. Each spleen was cut into two pieces, one was homogenized in PBS and from the other piece a single cell suspension was prepared. Further studies were carried out on these two samples.

Weight of spleen: At the time of killing the body weight of mouse was recorded. The spleen was removed. The weight of the spleen was represented as mg/100g body weight of the mouse.

Preparation of spleen cell culture: The spleen cells were teased out in cold MEM and a single cell suspension was prepared. The viable nucleated cell count as ascertained by trypan blue dye exclusion test was more than 95%.

Spleen cell proliferation assay: The cell suspension of spleen was adjusted to $2x10^6$ cells/ml in Eagle's MEM media supplemented with 10% FCS. Cell cultures were setup in 96 wells U- bottom microtitre plate in triplicate. Each well contain 0.2 ml cell suspension with or without 5 µg/ml. Concanavalin (Con) A. The cultures were incubated for 72 hrs at 37°C in presence of 5% CO_2. Five hours before the termination of the cultures, 1 µCi of [^{3}H]-Thymidine was added to each well. Cells were harvested and washed repeatedly with PBS (pH 7.2). Cell associated radioactivity was measured in Scintillation vials containing 5 ml. Scintillation fluid and were counted in Beta counter (Liquid Scintillation Fluid Analyser, TRI CARB 2900TR, Packard Biosciences Company). The data has been presented as mean value ±SD of counts per minute (cpm).The stimulation Index was calculated as ratio of the CPM to Con A treated cultures to that of media alone.

Preparation of spleen homogenate: Spleens were washed free of blood, homogenized in a tissue homogenizer giving a 10% solution (w/v) in chilled phosphate buffered saline (pH 7.2).The homogenate was centrifuged in the cold at 3000 rpm for 10 min. and clear supernatant was stored in small aliquots at –20°C. Normal mouse spleen homogenates were prepared similarly and used as control.

Cytotoxicity test: The cytotoxic activity of the spleen homogenate was tested using normal mouse spleen cells as target. The test was carried out in microtitre plates with 96 wells, using 0.1 ml. Volume of each preparation. The target

single cell suspension of normal mouse spleen was prepared as described before. 0.1 ml. Volume of cell suspension containing 2x 10^6 cell/ml was added in each well. After thorough mixing, the trays were kept at 4°C in a refrigerator for one hour. The trays were rocked every 10 min during the period of incubation. Then the cell viability was measured by MTT (3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Mosmann, 1983). Throughout the test all procedures were carried out at 4°C in ice bath.

MTT assay: After 1 hour incubation 20 ul of MTT solution (5 mg/ml) was added to each well and the plates were incubated at 37°C for 4 hours. After 4 hours incubation media was aspirated carefully with the help of syringe and 200 *u*l of DMSO was added to each well, and mixed thoroughly to dissolve the dark blue crystal. After waiting for a few minutes at room temperature to ensure that all crystals were dissolved, the plates were read on Micro ELISA Reader using a test wave length 570 nm. The MTT assay was done in triplicate and the mean value of % of non-viable cell ± as obtained in repeated experiments are presented.

Phagocytic activity of splenic macrophages: Spleen cell suspension layered on glass slides placed in Petridishes was incubated at 37°C for 2 h in an atmosphere of 5% CO_2. The glass-non-adherent cells were decanted gently. The glass-adherent cells were washed thrice with MEM and used (Chaturvedi *et al.*, 1982). The phagocytic activity of glass-adherent macrophages was assessed using latex particles (Cline and Lehrer, 1968). The latex particle suspension was added to the macrophages cell sheet and incubated for 1 h at 37°C in CO_2 incubator. The slides were washed to remove free latex particles and the cells were fixed with methanol and stained by the Giemsa technique. The slide was then examined under an oil immersion lens (100x) for cell containing intracellular latex particles. On each slide 200-300 cells were counted and the data presented percentage of phagocytic cells (Gulati *et al.*, 1982).

RESULTS

Effects on weight of spleen: The weight of spleen in the control normal mice was 518+19 mg/100 gm body weight. The data presented in Figure IV.2.1 shows a gradual reduction in the weight of spleen from 3 to 9 weeks after drinking Cr(VI). The weight of spleen after 3 weeks was 397±26mg/100 gm body weight while that after 9 weeks was 255±29 mg/100 gm body weight. Thus a 51% reduction in the weight of spleen was noted by drinking Cr(VI) for 9 weeks.

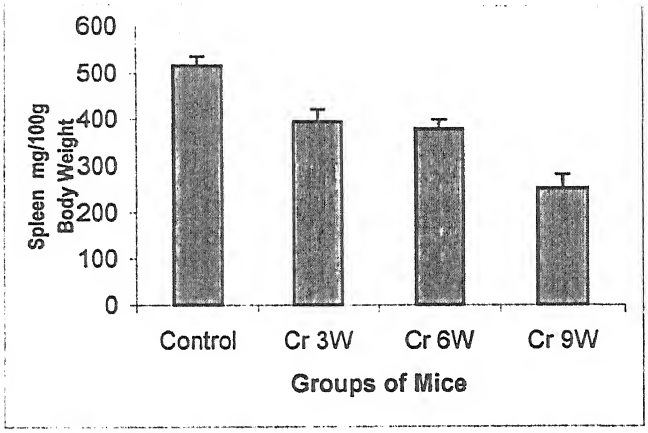


Figure IV.2.1: Weight of the spleens obtained from various groups of mice. Control, spleen from normal mice; Cr3W, mice drinking Cr(IV) for 3 weeks; Cr6W, mice drinking Cr(IV) for 6 weeks; Cr9W, mice drinking Cr(IV) for 9 weeks.

The data presented in Figure IV.2.2 compares the weight of the spleen in different groups. At 3 weeks of Cr water drinking the finding show a reduction in the weight of the spleen of normal mice inoculated with DV, the weight being 353±30 and 340±25 mg/100 g on the 4th and 8th day post-inoculation respectively. The reduction being 32% to 34%. The mice fed Cr(VI) for 3 weeks and inoculated with DV showed an increase in the wt. of spleen being 535±35 at the 4th day. The values were more or less similar to that of normal control mice .A similar finding was observed by feeding Cr for 6 weeks (Figure

IV.2.3) and 9 weeks (Figure IV.2.4). The increase observed in the weight of the spleen by inoculation of DV in Cr(VI) treated mice was minimal at 9 weeks .

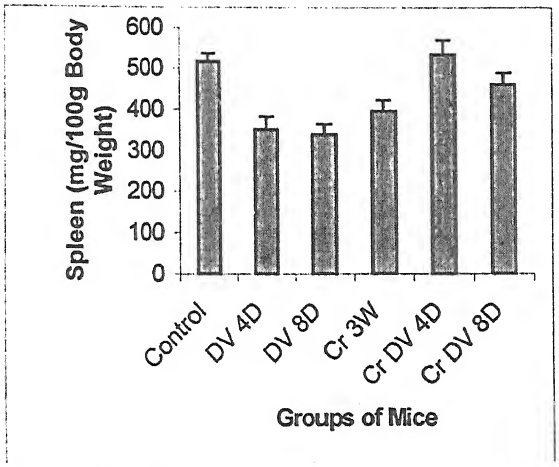


Figure IV.2.2: Weight of the spleens obtained from various groups of mice. Control, spleen from normal mice; DV4D, normal mice inoculated with dengue virus 4days post inoculation; DV8D, normal mice inoculated with dengue virus 8days post inoculation; Cr3W, mice drinking Cr(IV) for 3 weeks; CrDV4D, mice drinking Cr(IV) for 3 weeks inoculated with dengue virus day 4 post infection; CrDV8D, mice drinking Cr(IV) for 3 weeks inoculated with dengue virus day 8 post infection.

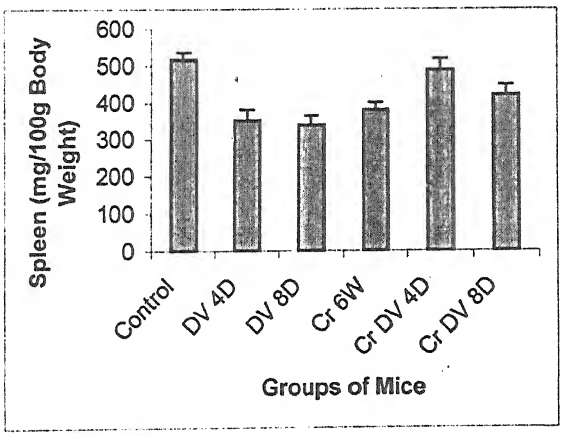


Figure IV.2.3: Weight of the spleens obtained from various groups of mice. Control, spleen from normal mice; DV4D, normal mice inoculated with dengue virus 4days post inoculation; DV8D, normal mice inoculated with dengue virus 8days post inoculation; Cr6W, mice drinking Cr(IV) for 6 weeks; CrDV4D, mice drinking Cr(IV) for 6 weeks inoculated with dengue virus day 4 post infection; CrDV8D, mice drinking Cr(IV) for 6 weeks inoculated with dengue virus day 8 post infection.

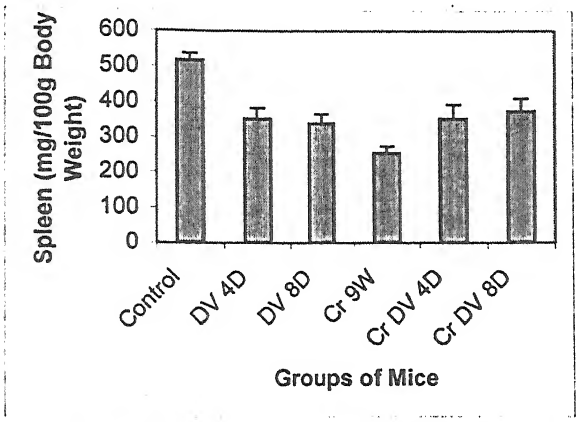


Figure IV.2.4: Weight of the spleens obtained from various groups of mice. Control, spleen from normal mice; DV4D, normal mice inoculated with dengue virus 4days post inoculation; DV8D, normal mice inoculated with dengue virus 8days post inoculation; Cr9W, mice drinking Cr(IV) for 9 weeks; CrDV4D, mice drinking Cr(IV) for 9 weeks inoculated with dengue virus day 4 post infection; CrDV8D, mice drinking Cr(IV) for 9 weeks inoculated with dengue virus day 8 post infection.

Effects on Cytotoxicity: The data presented in Figure IV.2.5 show the cytotoxic activity of the spleen homogenates in the different groups of mice. When the homogenate from the normal control mice was mixed with normal mouse spleen cells almost not cytotoxic activity was seen. The homogenate from DV infected mice spleen was 12 to 21%. On the other hand a higher cytotoxic activity was seen in the spleen homogenates obtained from Cr(VI) treated mice, the maximum being at 6 weeks when 66% cells was killed. Figure IV.2.6 compares the findings of the cytotoxic activity of the homogenates obtained from the spleen of mice drinking Cr(VI) for 3 weeks and inoculated with DV. It was observed that the cytotoxic activity was 58 and 52 % on the 4th and 8th day of post inoculation. After 6 weeks of drinking Cr(VI) water the cytotoxic activity was higher in chromium groups being 56 to 66% (Figure IV.2.7) The difference between the only Cr(VI) and Cr+DV groups was not present as seen at 3 weeks. The data presented in Figure IV.2.8 of the mice drinking Cr(VI) for 9 weeks is more or less similar to that at 6 weeks.

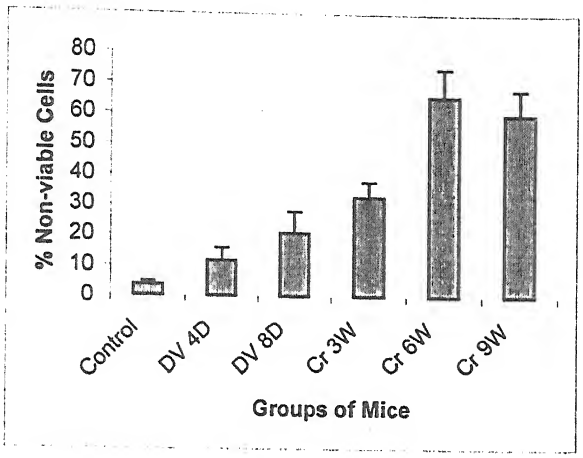


Figure IV.2.5: Cytotoxic activity of the homogenates of spleens obtained from various groups of mice. Control, spleen from normal mice; DV4D, normal mice inoculated with dengue virus 4days post inoculation; Cr3W, mice drinking Cr(IV) for 3 weeks; Cr6W, mice drinking Cr(IV) for 6 weeks; Cr9W, mice drinking Cr(IV) for 9 weeks

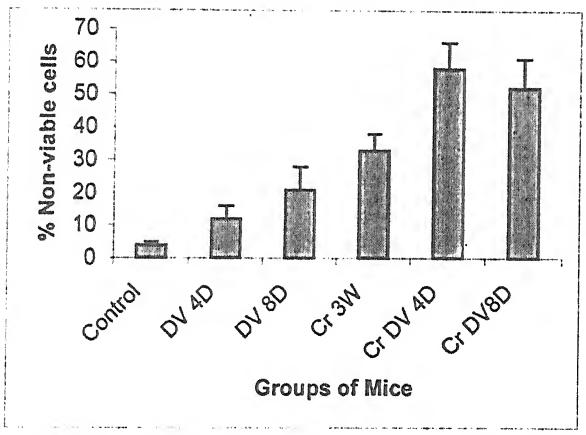


Figure IV.2.6: Cytotoxic activity of the homogenates of spleens obtained from various groups of mice. Control, spleen from normal mice; DV4D, normal mice inoculated with dengue virus 4days post inoculation; Cr3W, mice drinking Cr(IV) for 3 weeks; CrDV4D, mice drinking Cr(IV) for 3 weeks inoculated with dengue virus day 4 post infection; CrDV8D, mice drinking Cr(IV) for 3 weeks inoculated with dengue virus day 8 post infection

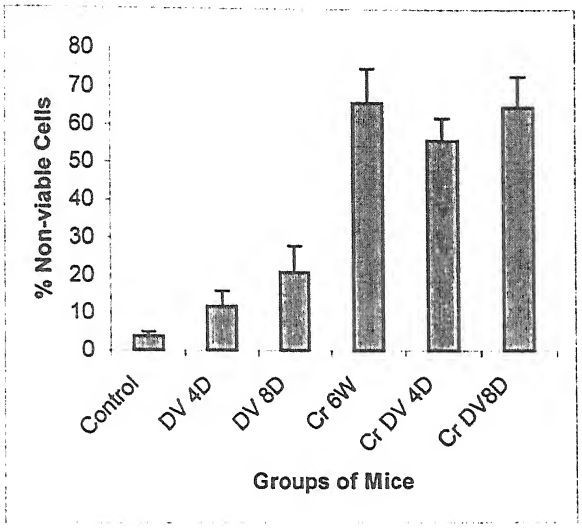


Figure IV.2.7: Cytotoxic activity of the homogenates of spleens obtained from various groups of mice. Control, spleen from normal mice; DV4D, normal mice inoculated with dengue virus 4days post inoculation; Cr6W, mice drinking Cr(IV) for 6 weeks; CrDV4D, mice drinking Cr(IV) for 6 weeks inoculated with dengue virus day 4 post infection; CrDV8D, mice drinking Cr(IV) for 6 weeks inoculated with dengue virus day 8 post infection.

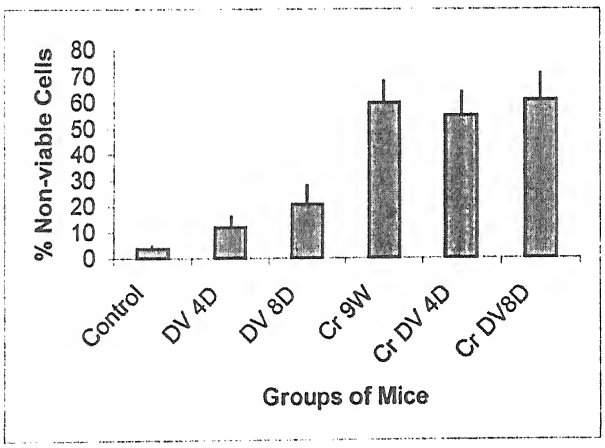


Figure IV.2.8: Cytotoxic activity of the homogenates of spleens obtained from various groups of mice. Control, spleen from normal mice; DV4D, normal mice inoculated with dengue virus 4days post inoculation; Cr9W, mice drinking Cr(IV) for 9 weeks; CrDV4D, mice drinking Cr(IV) for 9 weeks inoculated with dengue virus day 4 post infection; CrDV8D, mice drinking Cr(IV) for 9 weeks inoculated with dengue virus day 8 post infection.

Effects on phagocytic activity: The data presented in Figure IV.2.9 shows the phagocytic activity of the splenic macrophages in the different groups of mice. In normal control mice phagocytic activity of the splenic macrophages was 92±3%. When normal mice were inoculated with 1000 of LD_{50} of DV, phagocytic activity of splenic macrophages was reduced and became 65±6 and 45±8% at the 4th and 8th day post inoculation. On the other hand higher reduction in phagocytic activity was also seen in the splenic macrophages of Cr(VI) treated mice, the maximum reduction was observed at the 9th week when phagocytic activity was only 36±7% (Figure IV.2.9). A comparison of the phagocytic activity of the splenic macrophages of mice drinking Cr(VI) water for 3 weeks and inoculated with DV has been presented in Figure IV.2.10. It was observed that phagocytic activity was 63 and 52% on the 4th and 8th day post inoculation of DV. After 6 weeks of drinking Cr(VI) water and DV inoculation phagocytic activity was more or less similar to that at the 3rd week (Figure IV.2.11). After 9 weeks of drinking Cr(VI) water phagocytic activity of the splenic macrophages was significantly reduced and in mice inoculated with DV being 35±6 and 26±5% at the 4th and 8th day (Figure IV.2.12).

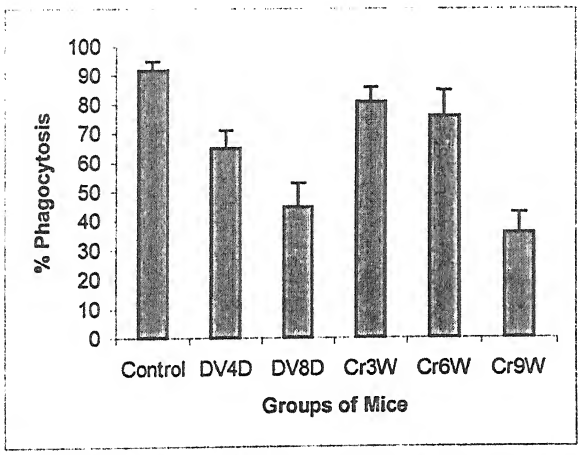


Figure IV.2.9: Phagocytic activity of the macrophages of spleens obtained from various groups of mice. Control, spleen from normal mice; DV4D, normal mice inoculated with dengue virus 4days post inoculation; Cr3W, mice drinking Cr(IV) for 3 weeks; Cr6W, mice drinking Cr(IV) for 6 weeks; Cr9W, mice drinking Cr(IV) for 9 weeks.

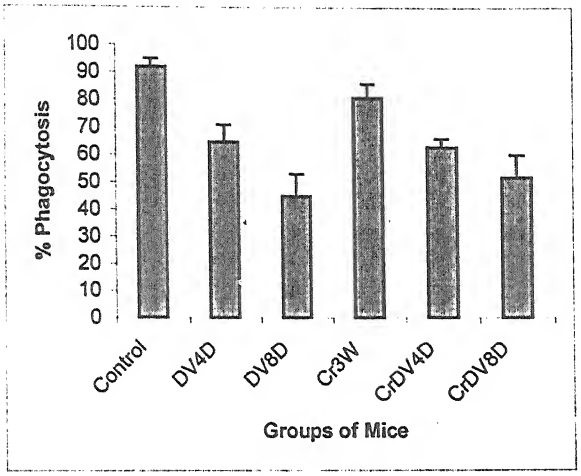


Figure IV.2.10: Phagocytic activity of the macrophages of spleens obtained from various groups of mice. Control, spleen from normal mice; DV4D, normal mice inoculated with dengue virus 4days post inoculation; Cr3W, mice drinking Cr(IV) for 3 weeks; CrDV4D, mice drinking Cr(IV) for 3 weeks inoculated with dengue virus day 4 post infection; CrDV8D, mice drinking Cr(IV) for 3 weeks inoculated with dengue virus day 8 post infection.

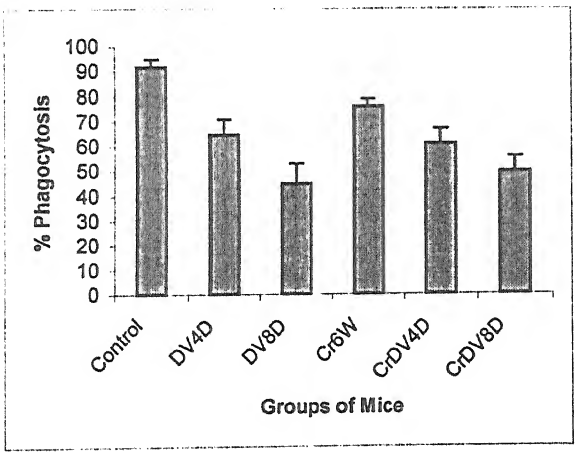


Figure IV.2.11: Phagocytic activity of the macrophages of spleens obtained from various groups of mice. Control, spleen from normal mice; DV4D, normal mice inoculated with dengue virus 4days post inoculation; Cr6W, mice drinking Cr(IV) for 6 weeks; CrDV4D, mice drinking Cr(IV) for 6 weeks inoculated with dengue virus day 4 post infection; CrDV8D, mice drinking Cr(IV) for 6 weeks inoculated with dengue virus day 8 post infection.

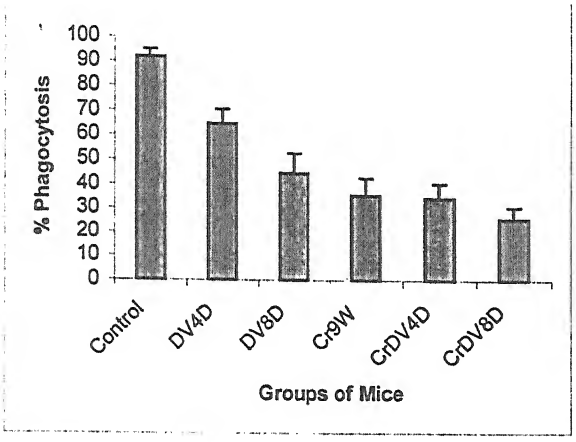


Figure IV.2.12: Phagocytic activity of the macrophages of spleens obtained from various groups of mice. Control, spleen from normal mice; DV4D, normal mice inoculated with dengue virus 4days post inoculation; Cr9W, mice drinking Cr(IV) for 9 weeks; CrDV4D, mice drinking Cr(IV) for 9 weeks inoculated with dengue virus day 4 post infection; CrDV8D, mice drinking Cr(IV) for 9 weeks inoculated with dengue virus day 8 post infection.

Effect on mitogenic stimulation

The findings presented in Figure IV.2.13 shows the mitogenic stimulation of the spleen cells in the different groups of mice. Stimulation was presented as thymidine uptate by spleen cells in counts per minute. When normal mice spleen cells were treated with mitogen the observed stimulation was 3500 cpm. When normal mice was inoculated with DV it was found that maximum proliferation was occurred at the 4^{th} day that was 10000 cpm but at the 8^{th} day it was decreased and returns to more or less similar to normal spleen cells. On the other hand after 9 weeks of drinking Cr(VI) water in mice, spleen cell proliferation was increased by two folds of normal mice spleen cells. When Cr(VI) treated mice was inoculated with DV proliferation observed less than Cr group but more than DV inoculated group.

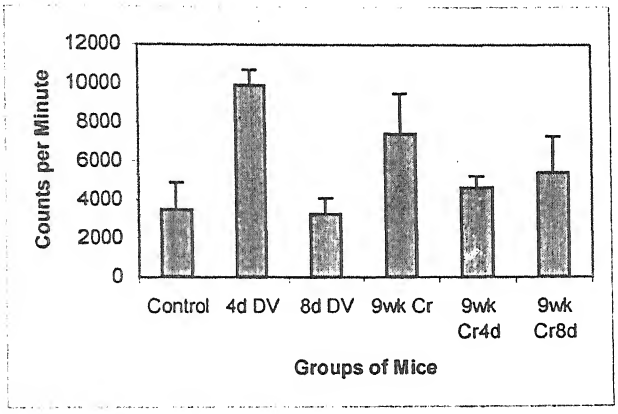


Figure IV.2.13: Effects of mitogenic stimulation of spleen cells from cells of different groups by Concanvalin A. Control, cells from normal mice; DV, cells from dengue virus inoculated mice; Cr, Cr (VI) fed mice.

DISCUSSION

The findings of the present study show a significantly reduction in the weight of spleen following oral exposure of mice to subtoxic doses of Cr(VI). The reduction in the weight of spleen was gradual with the increasing period of Cr (VI) feeding and was maximum at the 9th week, the reduction being 51%. The studies of Khangarot *et al.* (1999) and Arunkumar *et al.* (2000) conducted in fishes show similar results with the subtoxic doses of hexavalent chromium. A significant reduction (32 to 34%) in the weight of spleen was observed when normal control mice were inoculated with DV. It has been reported that during DV infection of mice the weight of spleen is markedly reduced associated with a sharp decline in the proportion of T-lymphocytes and macropgages in the spleen (Tandon *et al.*,1979a; Hotta *et al.*, 1981; Chaturvedi *et al.*; 1983). On the other hand when Cr(VI) fed mice were inoculated with DV the weight of spleen was increased as compared to that in mice fed Cr(VI) or inoculated with DV. The reason of this apparent increase in spleen weight appears to be due to the greater decrease in the body weight of mice. A summary of the comparison of the effects of Cr (VI) and DV on spleen is presented in Figure IV.2.14.

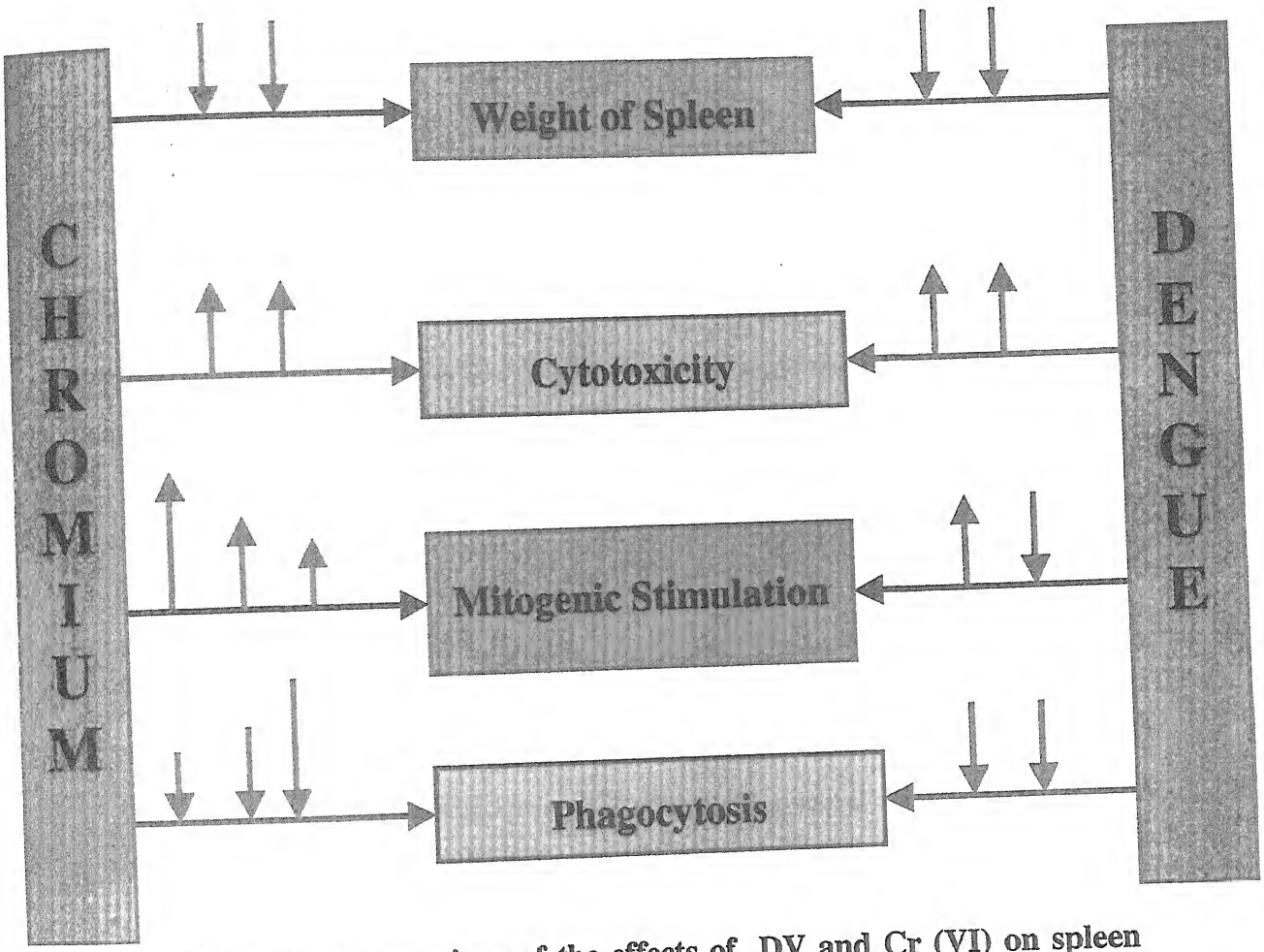


Figure IV.2.14: Comparison of the effects of DV and Cr (VI) on spleen and its cells.

During dengue virus infection the $CD4^+$ T cells produce a unique cytokine, Cytotoxic Factor (CF), in mice (mCF) and its homologue in man (hCF). mCF and hCF appear to be pathogenesis-related proteins, capable of reproducing DHF-like pathological lesions in mice, such as increased capillary permeability, cerebral edema, and blood leukocyte changes. CF is present in the homogenates prepared from the DV-infected mouse spleen and has the capacity to kill normal mouse spleen cells in one hour time (Chaturvedi *et al.*, 1991;1997; Mukerjee and Chaturvedi, 1995; Mukerjee *et al.*, 1997). The findings of the present study show that the cytotoxic activity of spleen homogenate of DV-infected mice was 12 to 23%. The cytotoxic activity of

spleen homogenates obtained from mice fed Cr(VI) for 3 weeks was 33±6% and that of Cr(VI) plus DV were 52 to 58%. Such summation of cytotoxic effect was not seen at 6 and 9 weeks as the cytotoxic activity of Cr(VI) fed mice was very high. A comparison of the effects of Cr(VI) and dengue virus infection on spleen is summarized in Figure IV.2.14. Cr(VI) is carcinogenic and mutagenic. Toxic effects of Cr (VI) in *in vivo* and *in vitro* are related to its intracellular fate. However, the mechanism of the Cr(VI)-induced cytotoxicity is not entirely understood. A series of *in vitro* and *in* vivo studies have demonstrated that Cr(VI) induces an oxidative stress through enhanced production of reactive oxygen species leading to genomic DNA damage and oxidative deterioration of lipids and proteins. A cascade of cellular events occur following Cr(VI)-induced oxidative stress including enhanced production of superoxide anion and hydroxyl radicals, increased lipid peroxidation and genomic DNA fragmentation, modulation of intracellular oxidized states, activation of protein kinase C, apoptotic cell death and altered gene expression (Bagchi *et al.*, 2001; de Neves *et al.*, 2001; Stohs *et al.*, 2001). Pritchard *et al.* (2000) also observed that certain hexavalent chromium compounds induce apoptosis as a predominant mode of cell death and showed that Cr(VI) induced apoptosis in Chinese hamster ovary (CHO) cells involves disruption of mitochondrial stability.

EXPERIMENT 3. DETOXIFICATION OF HEXAVALENT CHROMIUM BY CELLS OF IMMUNE SYSTEM

INTRODUCTION

Hexavalent chromium (Cr-VI) compounds are generally soluble over a wide pH range and have been shown to exert toxic and carcinogenic effects in humans and experimental animals (Norseth. 1986; Langard, 1988; Morris *et al.*, 1988; Antilla, 1990; Costa, 1997). In addition, Cr (VI) compounds also induce DNA damage such as DNA single-strand breaks and DNA-protein crosslinks *in vivo* and in cultured cells (Deflora *et al.*, 1990). Cr (VI) compounds are much more toxic than those of Cr (III) due to their higher solubility in water, rapid permeability through biological membranes and subsequent interaction with intracellular proteins and nucleic acids.

Chromium enters the body through the lungs, gastro-intestinal tract, and to a lower extent through skin (Hamilton and Wetterhahn, 1988). Inhalation is the most important route for occupational exposure, whereas non-occupational exposure occurs via ingestion of chromium containing food and water (Langard, 1982; Hertel, 1986). Most of the chromium absorbed by inhalation exposure, in comparison to the oral administration, is distributed in the lungs, liver, kidneys, RBC, plasma, spleen and bone marrow (Langard, 1982). All the ingested Cr (VI) is reduced to Cr (III) before entering in the blood stream (Kerger *et al.*, 1996). Cr (VI) enters into the cells through membrane anionic transporters while Cr (III) does not. Intracellular Cr (VI) is metabolically reduced to the ultimate Cr (III). It is therefore in the interest of the body to reduce the toxic Cr (VI) to less toxic form, the Cr (III). The cells of the immune system form a strong line of defence against foreign substances and the most common route of entry of chromium is through drinking water and food. The present study was, therefore, undertaken to investigate the capacity of different cells of immune system and intestine of Wistar rats to reduce Cr (VI).

METHODS AND MATERIALS

Animals: The study was carried out on Wistar rats weighing 200-250 g and maintained in the Animal House of the Institute.

Preparation of spleen cells: The rats were sacrificed by cervical dislocation and the spleens were removed aseptically. The spleen cells were teased out in cold MEM and a single cell suspension was obtained. The cells were washed and viable cell count done by trypan blue dye exclusion test (Chaturvedi *et al.*, 1978).

Preparation of splenic macrophages: Spleen cell suspension was incubated at 37°C for 2 h in an atmosphere of 5% CO_2 and the glass non-adherent cells were decanted gently. The glass-adherent cells were washed thrice with MEM and scrapped off with a policeman rod and resuspended as a single cell suspension. More than 95% cells were phagocytic (Chaturvedi *et al.*, 1982).

Separation of T and B lymphocytes: T and B lymphocytes were separated with glass wool and nylon wool columns by the technique of Julius *et al.*. (1973) and Trigio & Cudkowicz (1974). The effluent (T lymphocytes) and the eluted cells (B lymphocytes) were centrifuged separately and resuspended in cold MEM and the purity was tested as described elsewhere (Tandon *et al.*, 1979).

Preparation of peritoneal macrophages: The peritoneal cavity of rat was lavaged with 10 ml. of heparinized Eagle's minimum essential medium containing non-essential amino acids (MEM). The aspirated cells were layered in glass Petridish and incubated at 37°C in presence of 5% CO_2 for 2h. The glass non-adherent cells were removed by washing three times. The glass-adherent cells scrapped off with a policeman rod. Cells were resuspended in MEM and a single cell suspension was prepared (Chaturvedi *et al.*, 1982).

Preparation of thymocytes: The single cell suspension of thymocytes was prepared in MEM with 10% FCS as described by Debetto *et al.* (1988). The viable cell count was done by trypan blue dye exclusion test.

Estimation of chromium (VI) reduction by the cells: Potassium dichromate (hexavalent chromium; Cr (VI)) was of analytical grade and was purchased from M/S Qualigens, Lucknow. Chromium solution was prepared in MEM containing 10 or 25 µg/ml of chromium. $5x10^6$ cells were suspended in one-ml chromium solution and incubated at 37°C. At different time periods an aliquot was taken, the cells were removed by spinning and the reduction of Cr (VI) was assayed in the cell-free supernatant spectrophotometrically at 540 nm using diphenylcarbazide reagent (Uno and Anders, 1950). The experiments were setup in triplicate and were repeated thrice. The mean data ± SD has been presented as percentage reduction of Cr (VI).

RESULTS

Reduction of Cr (VI) by splenic lymphocytes: The data summarized in Figure IV.3.1 show that total spleen cells suspended in 10 µg/ml of Cr (VI) reduced 55±10% of it in 1 h time and the same level was maintained throughout the period of observation. The reduction by enriched subpopulation of B-lymphocytes was 18±5% at 1 h and gradually increased reaching peak of 40±8% reduction at 6 h of incubation. The least effective cells were enriched subpopulation of T-lymphocytes that reduced 10±2% of the Cr (VI) at 8 h.

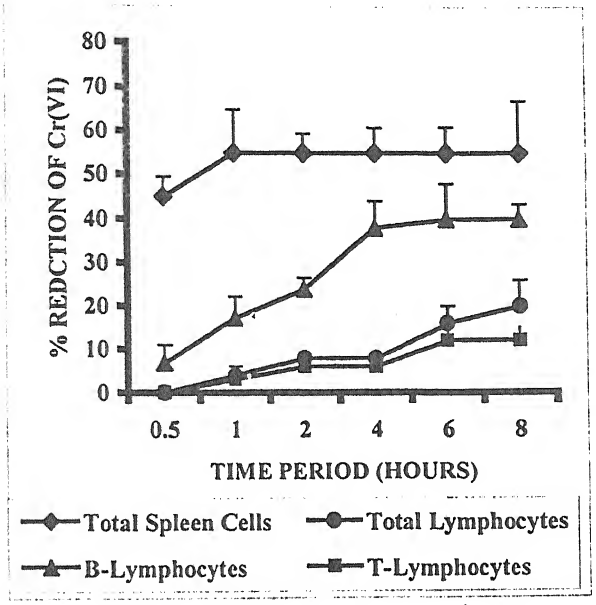


Figure IV.3.1: Reduction of Cr (VI) by rat spleen cells or its enriched subpopulations. A single cell suspension of normal rat spleen was prepared and enriched subpopulation of total lymphocytes were obtained by depleting macrophages with a glass-wool column and the T- and B-lymphocyte-enriched subpopulations were prepared with nylon-wool column. $5x10^6$ cells/ml were suspended in MEM containing 10 µg Cr (VI). At different time periods a group of sets were removed and Cr (VI) measured. The findings have been presented as mean value ± SD (bars) from repeated experiments as percentage reduction.

Reduction of Cr (VI) by macrophages: The macrophages obtained from the rat spleen and the peritoneal cavity were used. The finding presented in Figure IV.3.2 show that with 10 µg Cr (VI) the peak reduction of 22±5% was attained by splenic macrophages at 12 h while that by the peritoneal macrophages was similar, the peak being 24±7% at 12 h. This experiment was repeated using a higher dose of 25 µg chromium. A lower percentage of reduction (nearly half of the 10µg) was observed with the higher Cr (VI) concentration (Figure IV.3.2).

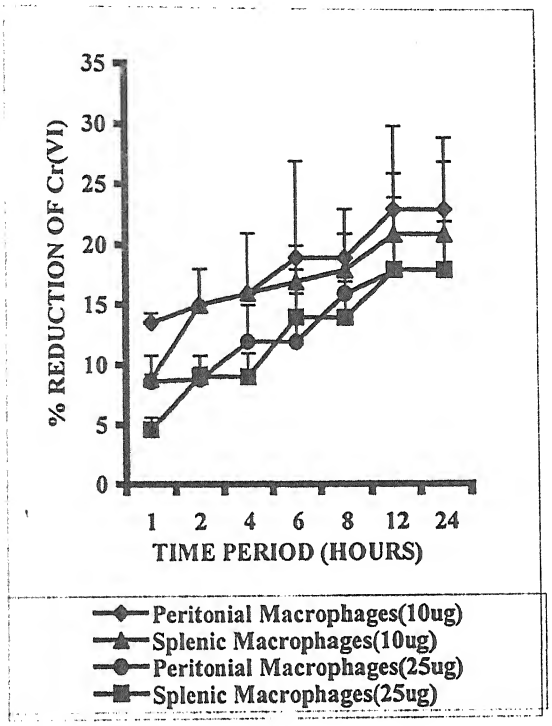


Figure IV.3.2: Reduction of Cr (VI) by macrophage-enriched cells obtained from rat peritoneal lavage cell and the spleen. The peritoneal lavage or the single cell suspension of spleen was layered in glass Petridish and incubated at 37°C for 2hours in presence of 5% CO_2. Then the adherent cells were scrapped off, counted and tested ($5x10^6$ cells/ml) as described in Figure 1 using 10 and 25 µg of Cr (VI).

Reduction of Cr (VI) by thymocytes: The total cell population obtained from the rat thymus was suspended in 10 and 25µg concentration of chromium and incubated for different time periods. The data presented in Figure IV.3.3 show that thymocyte gradually reduced Cr (VI) reaching peak reduction at 24h. The reduction was 54±15% with Cr (VI) concentration of 10µg and 28±8% in 25µg concentration (Figure IV.3.3).

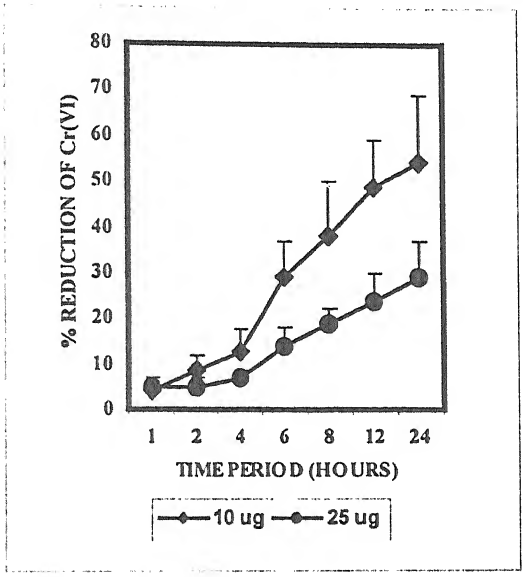


Figure IV.3.3: Reduction of Cr (VI) by rat thymocytes. Total thymus was removed from the rat and a single cell suspension was prepared and tested ($5x10^6$ cells/ml) as described in Figure 1.

DISCUSSION

The main finding of the present study is that various cells of the body differ in their capacity to reduce Cr (VI). Chromium is of significant importance in altering the immune response by immunostimulatory or immunosuppressive mode as shown by its effects on T and B lymphocytes, macrophages, cytokine production and the immune response that may induce hypersensitivity reactions (Shrivastava *et al.*, 2002). Cells of the immune system form the first line of defense in the body. These cells differed vastly in their capacity and the time taken to reduce Cr (VI). The most effective were the total spleen cells (55±10%) and thymocytes (54±15%), followed by B-lymphocyte enriched subpopulation of spleen (40±8%) and least were T-lymphocytes (10±2%) while macrophage reduced 24±7% of the Cr (VI). DeFlora *et al.* (1997) has reported similar findings with lung alveolar macrophages.

Cr (VI) enters into the cells through membrane anionic transporters but Cr (III) does not. While detoxifying Cr (VI), the cells themselves may get damaged. Chromium-induced damage to DNA both in the gastric mucosa cells and lymphocytes has been studied by comet assay and the effects were found to be similar in both (Blasiak *et al.*, 1999; Trzeciak *et al.*, 2000). Some of the important factors in determining the biological outcome of chromium exposure include the bioavailability, chemical speciation and solubility of chromium compounds, intracellular reduction, and interaction of chromium with DNA. Intracellular Cr(VI) is metabolically reduced to the ultimate Cr(III). Cr(VI) does not react with macromolecules such as DNA, RNA, proteins and lipids. However, both Cr(III) and the reductional intermediate Cr(V) are capable of co-ordinate, covalent interactions with macromolecules. A series of *in vitro* and *in vivo* studies have demonstrated that Cr(VI) induces an oxidative stress through enhanced production of reactive oxygen species leading to genomic DNA damage and oxidative deterioration of lipids and proteins. A cascade of cellular events occur following Cr(VI)-induced oxidative stress including enhanced production of superoxide anion and hydroxyl radicals, increased lipid peroxidation and genomic DNA fragmentation, modulation of intracellular oxidized states, activation of protein kinase C, apoptotic cell death and altered gene expression (Shrivastava *et al.*, 2002; Bagchi *et al.*, 2001; Stohs *et al.*, 2001).

Further, Cr(VI) is reduced to variable degree in different tissues and organs like, peripheral lung parenchyma, bronchial tree, kidney, testis, stomach, spleen, plasma and adrenals *etc.* (Deflora, 2000). The present study is unique in the sense that mostly single isolated cells have been tested. The reduction of Cr(VI) to Cr(III) results in the formation of reactive intermediates that contribute to the cytotoxicity, genotoxicity, and carcinogenicity of Cr(VI)-containing compounds. These mechanisms of reduction of Cr(VI) explain the

lack of genotoxicity, carcinogenicity, and induction of other long-term health effects of chromium (VI) by the oral route.

EXPERIMENT 4. DETOXIFICATION OF HEXAVALENT CHROMIUM BY CELLS OF INTESTINES

INTRODUCTION

Chromium enters the body through the lungs, gastro-intestinal tract, and to a lower extent through skin (Hamilton and Wetterhahn, 1988). Inhalation is the most important route for occupational exposure, whereas non-occupational exposure occurs via ingestion of chromium containing food and water (Langard, 1982; Hertel, 1996). Regardless of route of exposure Cr (III) is poorly absorbed whereas Cr (VI) is more readily absorbed (Hamilton and Wetterhahn, 1988). Further, absorption of Cr (VI) is poorer by oral route therefore, it is not very toxic when introduced by the oral route (Deflora *et al.*, 1997). All the ingested Cr (VI) is reduced to Cr (III) before entering in the blood stream (Kerger *et al.*, 1996). Cr (VI) enters into the cells through membrane anionic transporters while Cr (III) does not. Intracellular Cr (VI) is metabolically reduced to the ultimate Cr (III). It is therefore in the interest of the body to reduce the toxic Cr (VI) to less toxic form, the Cr (III). The cells of the immune system form a strong line of defence against foreign substances and the most common route of entry of chromium is through drinking water and food. The present study was, therefore, undertaken to investigate the capacity of different cells of intestine of Wistar rats to reduce Cr (VI).

METHODS AND MATERIALS

The study was carried out on Wistar rats using techniques described in Experiment 1.

Isolation of intestinal epithelial cells of various differentiation stages from Crypt to Villus: Over night fasted rats were sacrificed by cervical dislocation and decapitation. Small intestine was removed and flushed gently with normal saline containing 1.0 mM dithiothretol. Intestinal epithelial cells were prepared along crypt to villus axis on gradient of differentiation according to Weiser

(1973). In brief, cecal end of the intestine was ligated and solution 'A' containing 1.5 mM KCl, 96 mM NaCl, 27mM sodium citrate, 8 mM KH_2PO_4 (pH 7.3) was filled after clamping the other end with artery forceps. The intestine was then immersed in solution 'A' and incubated at 37°C for 15 min in a constant water bath. After incubation the intestine was emptied and fluid discarded. The intestine was now filled with solution 'B' containing 1.5 mM EDTA and 5 mM dithiothretol in PBS (pH 7.2) and immersed in solution 'B' for incubation. After 4 min incubation, the contents were emptied into a plastic centrifuge tube to recover the first epithelial cell population. The intestine was filled with solution 'B' for different time intervals and the process was repeated for several times to collect cell population of differentiation stages. Cell population were centrifuged at 900 g for 5 min and washed twice with 4 mM EDTA solution containing 15 mM β-mercaptoethanol (pH 7.4) to remove phosphate buffer. For this experiment, epithelial cells in their sequence of dissociation from the intestine were pooled into three fractions on the basis of their protein content and alkaline phosphatase activity as described by Panini *et al.*. (1979). These fractions were designated as Upper Villi, Middle Villi and Crypt respectively. From each fraction $5x10^6$ cells/ml were used in the tests.

Experiments on intestinal cells *in situ*: Laparotomy on each rat was performed by midline incision under light ether anaesthesia. The intestine was washed with normal saline, using a syringe and a blunt needle, through two small cuts. One was made slightly distal to the duodeno-jejunel junction and another at the distal end of ileum. After washing, the opening was ligated and 10 cm length loops were prepared from the upper end of intestine using sterile threads (Rastogi *et al.*, 1988). Test solution was administered into the loops through proximal opening, which was then immediately ligated. Control loops contained normal physiologic saline solution. The whole intestine was kept *in situ* and the abdomen stitched immediately. Proper breathing and anaesthesia of the animal was maintained throughout the experiment. Loops were removed

after 30 min incubation time, gently blotted on filter paper, and the contents were drained into graduated tubes. The luminal fluid was made up to desired volume and centrifuged at 500 *g* for 5 min to remove any intestinal debris.

Estimation of chromium (VI) reduction by the cells: The experiments were setup in triplicate and were repeated thrice as described in Experiment 1. The mean data ± SD has been presented as percentage reduction of Cr (VI).

RESULTS

Reduction of Cr (VI) by intestinal cells *in vitro*: Cells obtained from upper and middle villus and the crypts of the intestine were studied for their capacity to reduce Cr (VI). The findings summarized in Figure IV.4.1 shows that the reduction was 85±12 to 100% by the cells from the Upper Villus using the two concentrations of Cr (VI). The reduction was minimal with the cells from the crypts while that by the cells from the Middle Villus was 55±12 to 72±18% (Figure IV.4.1).

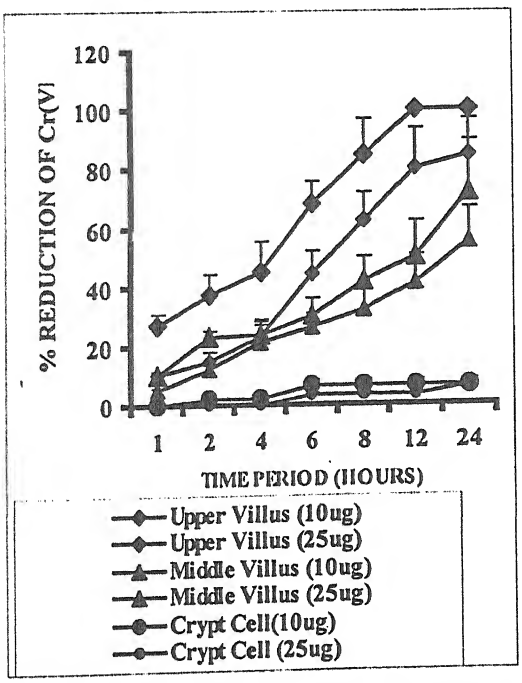


Figure IV.4.1: Reduction of Cr (VI) by rat intestinal cells. Rat intestines were removed and different types of cells were removed and a single cell suspension was prepared and tested ($5x10^6$ cells/ml) as described in Materials & Methods.

Reduction of Cr (VI) by intestinal cells *in situ*: The intestinal loops were blotted on filter paper, and the contents were drained into graduated tubes. The luminal fluid was made up to desired volume and centrifuged to remove any intestinal debris. The Data summarized in Figure IV.4.2 shows that the reduction of Cr (VI) was 100%, 90±7% and 90±10% with 10, 50 and 100 *u*g Cr (VI) respectively.

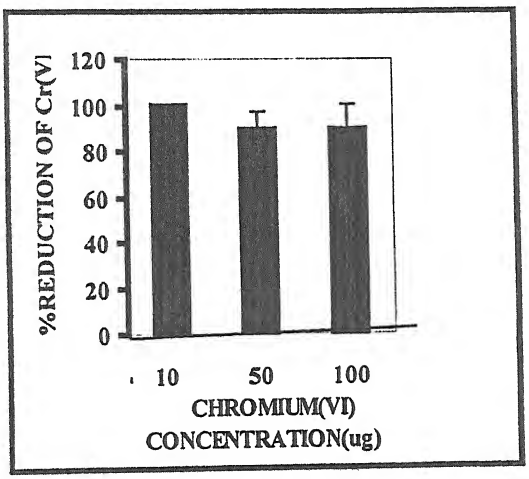


Figure IV.4.2: Reduction of Cr (VI) by rat intestinal loop *in situ*. Rat intestines were exteriorized; lumen washed and different doses of Cr (VI) injected in lumen and Cr (VI) reduction tested as described in Materials & Methods.

DISCUSSION

The main finding of the present study is that among the isolated rat cells tested, peak reduction of 100 % was seen by Upper Villus cells of intestine while the least (4%) was seen with the Crypt cells of the intestine, the other cells were in between. Chromium enters the body through the lungs, gastro-intestinal tract, and to a lower extent through skin (Hamilton and Wetterhahn, 1988). The most important route for non-occupational exposure is via ingestion of chromium containing food and water. Regardless of route of exposure Cr (III) is poorly absorbed whereas Cr (VI) is more readily absorbed (Langard, 1982; Hertel, 1986; Kerger *et al.*, 1996). Further, Cr (VI) is not very toxic when introduced by oral route and it was thought to be due to poorer absorption

by oral route. But our findings show that the reason was efficient detoxification of Cr (VI) by reduction in the intestines. The most efficient reduction (100%) of Cr (VI) was observed by the whole intestinal loop. A number of components in the intestines may be responsible for such efficient handling of Cr (VI). When introduced by the oral route, Cr (VI) is efficiently reduced by Upper Villus cells (100%), the Middle Villus cells (72±18%) and the Crypt cells (4%) of the intestine as shown in the present study. The intestinal bacterial flora, including *E. coli*, *Lactobacillus* sp. *Pseudomonas* sp. and *Staphylococcus* sp. have been found to be efficient reducer of Cr (VI) (Personal unpublished data). The findings of the present study may also suggest that whatever Cr (VI) crosses over the intestinal wall can be efficiently reduced by the lymphocytes and macrophages (as described in Experiment 1) present in the lymphoid follicles of the intestinal wall and also in the lymph nodes of the omentum. Our findings find support in the study of DeFlora *et al.* (1997) who have shown that saliva and gastric juice and sequestration by intestinal bacteria, blood, liver are efficient Cr (VI) reducers. This is further supported by a number of studies where chronic oral exposure to Cr (VI) exhibited no adverse effects (Mackenzie *et al.*, 1958; USEPA 1996; Mirsalis *et al.*, 1996). The present study is unique in the sense that mostly single isolated cells have been tested. The reduction of Cr (VI) to Cr (III) results in the formation of reactive intermediates that contribute to the cytotoxicity, genotoxicity, and carcinogenicity of Cr (VI)-containing compounds. These mechanisms of reduction of Cr (VI) explain the lack of genotoxicity, carcinogenicity, and induction of other long-term health effects of chromium (VI) by the oral route.

EXPERIMENT 5. DETOXIFICATION OF HEXAVALENT CHROMIUM BY INTESTINAL MICROFLORA

INTRODUCTION

The ingested Cr (VI) is reduced to Cr (III) before entering the blood stream. Cr (III) is unable to enter into the cells but Cr (VI) enters through membrane anionic transporters (USEPA, 1984; Kerger *et al.*, 1996). Cr (VI) is highly toxic to all forms of living organisms and is mutagenic in bacteria (Losi *et al.*, 1994). The presence of chromate in the environment inhibits most microorganisms but also promotes the selection of resistant bacteria. Cr (VI) compounds are markedly effective than those of Cr (III) due to their high solubility in water,' rapid permeability through biological membranes and subsequent interaction with intracellular protein and nucleic acids. The processes by which the microorganisms interact with the toxic metals enabling their removal/and recovery are biosorption, bioaccumulation and enzymatic reduction. Microorganisms have evolved resistance mechanism to select resistant variants to deal with metal toxicity as the result of exposure to metal contaminated environments, which cause co-incidental selection for resistant factors for antibiotics and heavy metals. Some bacteria present in water and soil develop resistance to chromium on exposure to Cr-containing effluent in their environment (Ohtake *et al.*, 1987; Wang *et al.*, 1990; Viti *et al.*, 2003). These bacteria reduce Cr (VI) into Cr (III) and minimize the adverse effects of Cr (VI) on their growth (Yamamoto *et al.*, 1993). In Experiment 1 I have reported that the different cells in the body differ vastly in their capacity to reduce Cr (VI), the most efficient being the intestines due to the presence of a variety of cells (Shrivastava *et al.*, 2003). The intestines have a huge population of bacteria and the caecum habours the largest number of 10^{10} to 10^{12} bacteria/g of intestinal contents (Siman and Gorbach, 1986). Further, some of the bacteria bioaccumulate large quantity of Cr and bring down the residual concentration of Cr (VI) in 24 h (Srinath *et al.*, 2002). Thus, bacteria may play an important

role in protecting body from the toxicity of ingested chromium. The bacteria present naturally in soil and water-bodies are exposed to Cr through contamination with industrial effluents, especially from tannery *etc.* The resident bacterial flora of the gastro-intestinal tract is exposed to Cr through ingestion of water and food contaminated with Cr. Several studies have been done to investigate the effect of Cr on isolated soil and water bacteria resistant to chromium but the literature on the effect of Cr on resident gut microflora is scarce (Losi *et al.*, 1994; Ohtake *et al.*, 1987; Srinath *et al.*, 2002; Francisco *et al.*, 2002; Viti *et al.*, 2003). It was, therefore, considered important to investigate the effect of chronic ingestion of chromium on the resident gut microflora of Wistar rats. I report here significant alterations in the response of facultative bacteria, *Lactobacillus* sp., *Pseudomonas* sp. and *Escherichia coli* of the rat gut to chromium.

MATERIALS AND METHODS

Preparation of stock solution of hexavalent chromium: Standard solution of 1000-ppm strength of chromium (VI) was prepared by dissolving $K_2Cr_2O_7$ in water.

Chromium (VI) treatment of Rats: Wistar rats weighing 200-250 g and maintained on pellet diet in the animal house of this Institute were used. One group comprising of 6 rats were given *ad-lib* drinking water containing 10 ppm of Cr (VI) for 10 weeks. These rats were designated as Cr-stressed. The second group of 6 rats was given plain water.

Isolation and Identification of resident gut microflora: At the end of 10th week all the animals were sacrificed. The abdomen was opened with full aseptic precautions. The caecum was located and injected with 5.0 ml of sterilized phosphate buffered saline (PBS) from one end. After 2 min. a small nick was made on the caecum and the fluid was collect in a sterilized Petridish. One loopful of the caecal fluid was streaked out on nutrient agar plate. The

caecal fluid from the Cr-stressed rats was streaked on nutrient agar plates containing 10 mg/L concentration of Cr (VI). After 24 h incubation the morphology of different bacterial colonies was recorded and the smears were stained with Gram's stain. The bacteria were identified on the basis of biochemical reactions according to the Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1989). The bacteria selected for further study from the two groups of rats were *Escherichia coli*, *Pseudomonas* sp. and *Lactobacillus* sp.

Growth pattern of normal and Cr-stressed intestinal microflora in presence of Cr (VI): Growth curves of bacterial isolates were prepared as described. A single colony of each isolated bacteria was subcultured in 5.0 ml. of peptone water. Growth phase of the bacteria were obtained and about $5x10^7$ bacterial cells were suspended in 100 ml. of peptone water containing various concentrations of Cr (VI). One control was also included for each bacterium without containing Cr (VI). The Optical Density of an aliquot of the culture was measured at 610 nm at 0, 2, 4, 6, 8,10, 12, 24 and 30 hrs after incubation. The tests were setup in triplicate and the mean value from all the animals of the group have been presented. The concentration of viable cells was determined by plating 100 µl of appropriately diluted culture on to nutrient agar and incubating the plates at 35°C for 24 h.

Curing of Plasmid: Plasmids of all the strains were cured by treatment of the bacterial cultures with acridine orange (125 µg/ml) at 42°C for 24 h as described else where (Dhakephalkar *et al.*, 1996).

Estimation of chromium (VI) reduction by the bacteria: Potassium dichromate (Cr (VI)) was of analytical grade and was purchased from M/S Qualigens, India. Chromium solutions were prepared in Peptone water containing 10, 25, 50 or 100 mg/L of Cr (VI). Growth phase of the bacteria were obtained and about $5x10^7$ bacterial cells were suspended in 100ml Peptone water with or without various concentrations of Cr (VI). They were

incubated at 37°C on a shaker at 100 rpm. At different time periods an aliquot was taken and the cells were removed by spinning at 8000g for 10 min. The reduction of Cr (VI) was assayed in the cell-free supernatant spectrophotometrically at 540 nm using diphenylcarbazide reagent (Urone, 1955). The experiments were setup in triplicate. The mean data ± SD has been presented as percentage reduction of Cr (VI).

Determination of minimal inhibitory concentration of Cr(VI): The minimal inhibitory concentration (MIC) of chromium at which no colony growth occurred was determined by broth agar dilution method (Luli *et al.*, 1983). The bacteria were inoculated into 25 ml peptone water (HiMedia, India) consisting of 1.0% (w/v) peptone and 5.0% (w/v) NaCl in conical flasks and incubated at 28°C at 150 rpm to achieve log phase cultures. Nutrient agar plates containing different concentrations (50 to 200 mg/L) of Cr (VI) were inoculated from the exponential growing cultures of each bacterial strain. These plates were incubated at 37°C for 48 h. The lowest concentration of Cr (VI) at which no bacterial growth occurred was considered as MIC.

Determination of antibiotic sensitivity: All the strains of bacteria isolated from the two groups of rats were tested for antibiotic sensitivity following the National Committee for Clinical Laboratory Standard (NCCL) disc diffusion method. Sensitivity of different cured strains to various antibiotics was also tested. The following antibiotic discs were used: Tetracycline (30 µg), Cotrimazol (25 µg), Trimethophorin (5 µg), Chloremphenicol (30 µg), Streptomycin (30 µg), Gentamycin (10 µg), Nalidixic acid (30 µg), Ampicillin (50 µg) and Kanamycin (30 µg).

Statistical analysis: Student's t-test was used for statistical evaluation of the data. A p value of less than 0.05 was considered significant.

RESULTS

Growth profile of the bacteria: The data presented in Figure IV.5.1 compares the growth profiles of the three bacteria isolated from the gut of normal and Cr-stressed rats grown in media without (Control) or with various concentrations of Cr (VI). The presence of the higher concentration of 100 mg/L of Cr (VI) in the growth media was detrimental to the growth of all the groups of bacteria (Figure IV.5.1). The growth profile of *E. coli* showed no significant difference in the growth rate of the bacteria obtained from the normal control rats and the Cr-stressed rats.

On the other hand, *Pseudomonas* and *Lactobacilli* obtained from the Cr-stressed rats tolerated the presence of Cr (VI) in the milieu much better than those from the normal rats; the growth in the Cr-stressed was much more. This point is reflected clearly in Figure IV.5.2, which shows the increase in the growth of Cr-stressed bacteria as compared to the normal rats at 24h. It was observed that the growth of *Lactobacillus* obtained from the Cr-stressed animals was 180±10% higher as compared to the *Lactobacillus* obtained from the unstressed rats. Cr-stressed bacteria grown in presence of 10 mg/L Cr (VI) revealed 230±25% increase in growth in comparison to the unstressed normal control ($p \leq 0.001$). In presence of 25 and 50 mg/L Cr (VI), the increase in growth of Cr-stressed bacteria was 150±26 and 102±20%, respectively. The increase in growth of control *Pseudomonas* obtained from Cr-stressed rats was most marked being 260±30% as compared to the unstressed controls. Furthermore, cells grown in presence of Cr (VI) revealed a concentration-dependent increase in growth, which was up to 320±17% with 50 mg/L ($p \leq 0.001$).

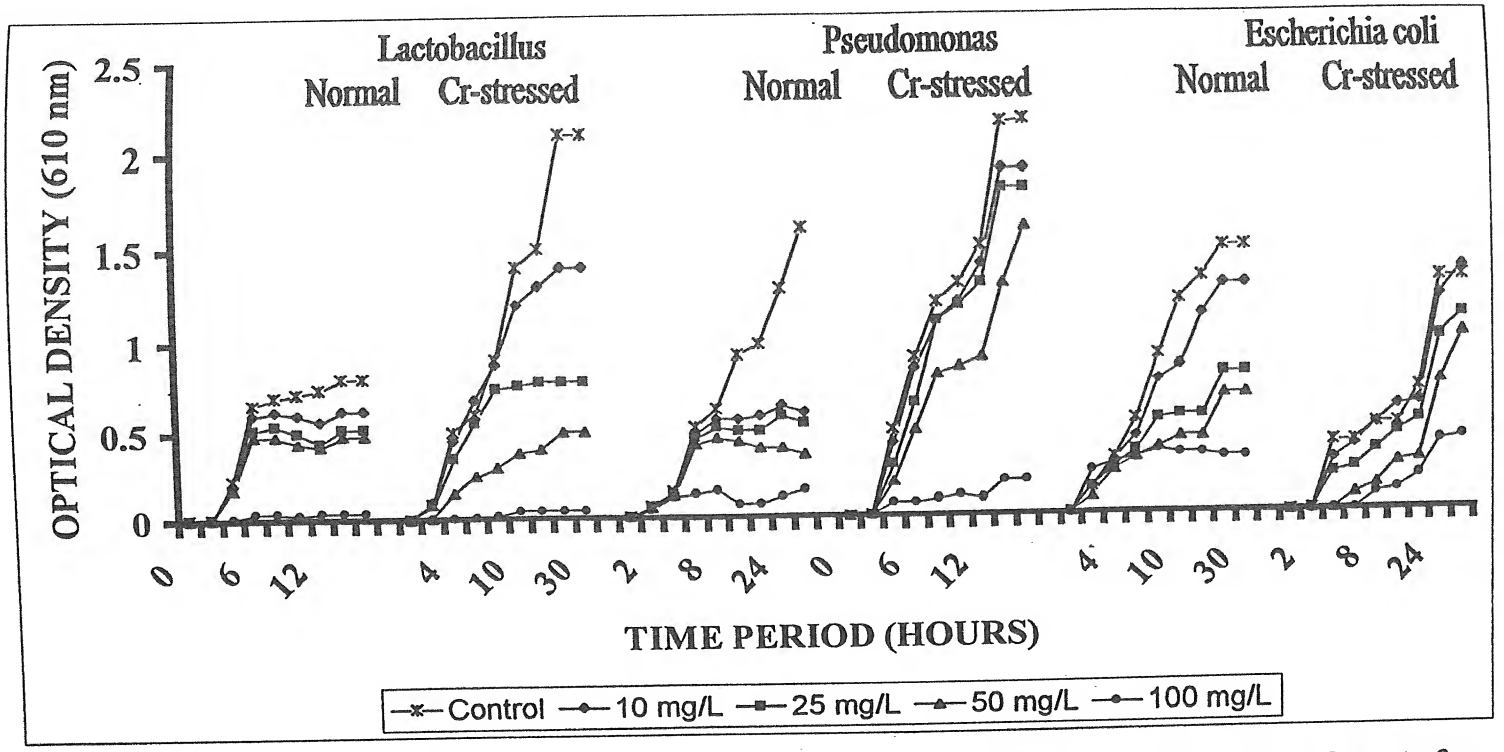


Figure IV.5.1: Figure 1: Comparison of the growth profile of the resident bacteria obtained from the gut of normal and Cr-stressed rats. Rats were divided into two groups each of 6 rats. One group was fed ad lib water containing 10 ppm of Cr (VI) and the second group received plain water. The rats were sacrificed after 10 weeks with aseptic precautions and the intestinal bacteria were isolated. The growth profile of the bacteria from the two groups of rats was studied in presence of various concentrations of Cr (VI). The Controls were grown in media without Cr (VI). The data has been presented as mean value from different rats. SD has not been shown to avoid overcrowding. Variance was within a limit of 10-15%.

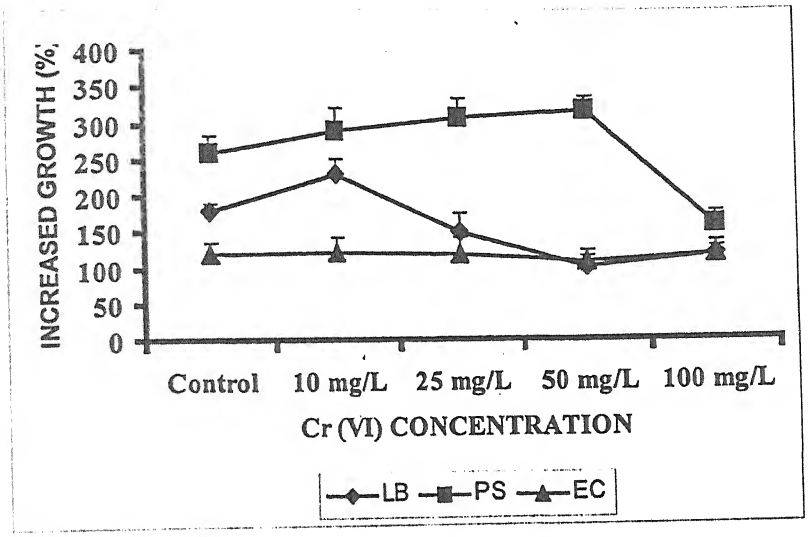


Figure IV.5.2: Increase in the growth of Cr-stressed bacteria in comparison with that from the normal rats at 24h. Data from Figure 1 was analyzed to get the extent of increase in growth. The data has been presented as mean value±SD of the % increase. Control, bacteria grown without Cr in the medium.

Further analysis of the data revealed a significant reduction in doubling time of Cr-stressed *Pseudomonas* and *Lactobacillus* throughout the growth period as compared to their respective bacteria from the normal rats (data not shown). Simultaneous comparison of Cr-stressed bacteria with respective normal bacteria when grown in presence of lower Cr concentrations also showed similar decrease in doubling time. Likewise, significant increase in number of generations was also evident in Cr-stressed bacteria. However, these changes were not observed in case of Cr-stressed *E.coli* (data not shown). The results of specific growth rate, doubling time and number of generations of Cr-stressed and bacteria from normal animals indicate bacterial specificity with respect to Cr-stress.

Reduction of Cr (VI) by the bacteria: The data presented in Figure IV.5.3 show the profile of reduction of various concentrations of Cr (VI) by the three

bacteria obtained from the normal control and the Cr-stressed rats. A concentration and time dependent decline in the Cr (VI) reduction in both cases, normal and Cr-stressed, were apparent. However, more significant decline in the capacity to reduce Cr (VI) in *Pseudomonas* and *Lactobacillus* obtained from Cr-stressed rats were observed as compared to their respective normal bacteria. *E.coli* on the other hand showed comparatively less Cr-reduction capacity. Overall data throughout the growth phase from Cr-stressed bacteria revealed that the effects were most marked with *Pseudomonas* and least with *E.coli*.

Further analysis of percent decrease in Cr (VI) reduction capacity of Cr-stressed bacteria in comparison to normal rat bacteria at the end of 6 and 24 h has been shown in Figure IV.5.4. At the end of 6 h, loss of capacity to reduce Cr (VI) by the *Pseudomonas* from the Cr-stressed rats was 48±7% at 10 mg/L concentration and the loss was 100% with 50 mg/L or higher concentrations. The least effected were *E. coli* where the loss of capacity to reduce Cr (VI) was 21±6 to 53±9% with various concentrations of Cr (VI). The *Lactobacilli* were found to be in between (Figure IV.5. 4A). After 24 h of incubation the pattern was changed with respect to *Pseudomonas*; the loss of capacity to reduce Cr (VI) with 50 and 100 mg/L concentrations was 64±15 and 56±12%, respectively. This showed recovery of the capacity to reduce Cr (VI) with increasing period of incubation.

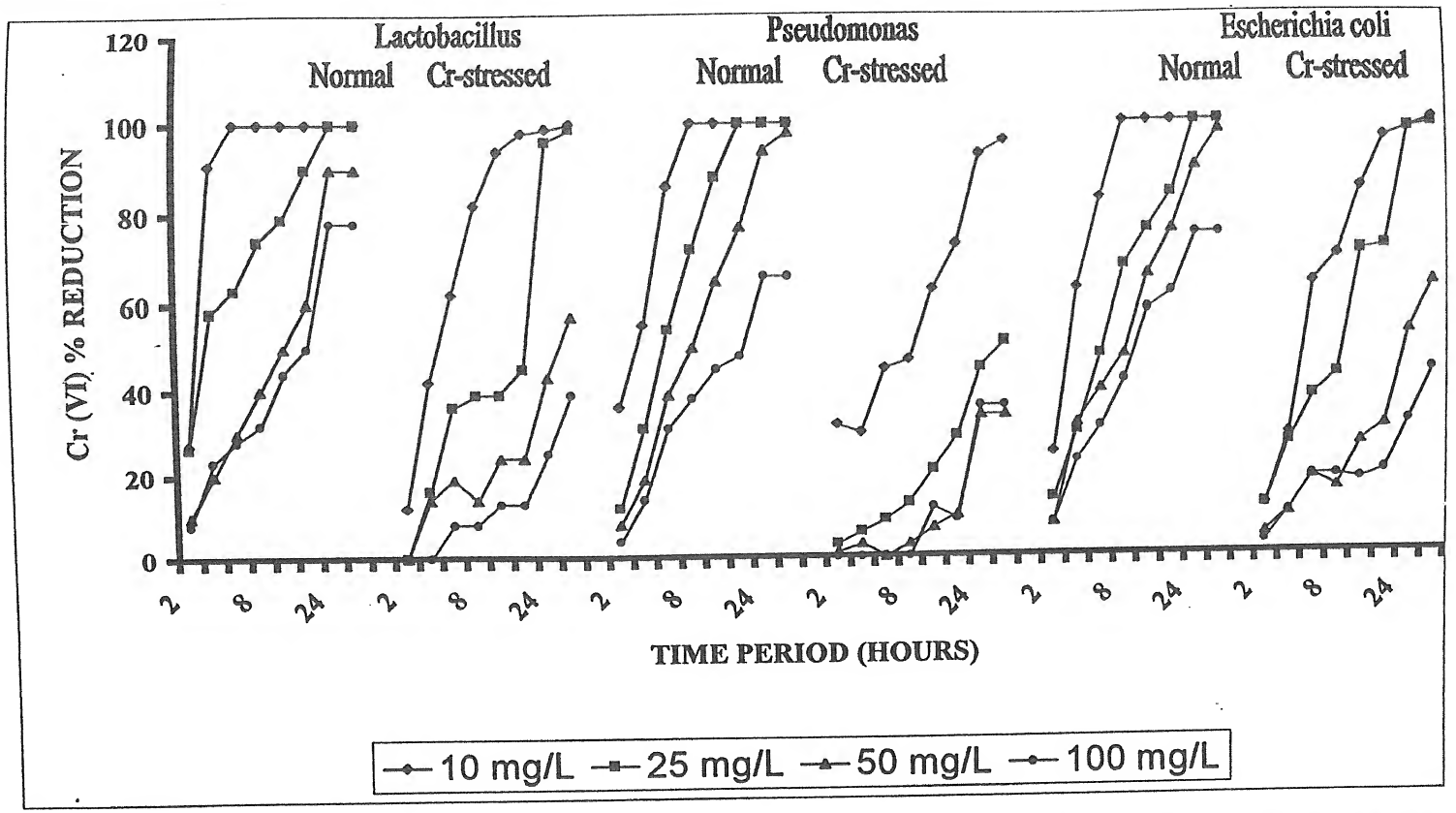


Figure IV.5.3: Reduction of Cr (VI) by the resident bacteria obtained from the gut of normal and Cr-stressed rats as stated in Figure IV.5.1. At different time periods a group of sets were removed and Cr (VI) measured. The findings have been presented as mean value ±SD from repeated experiments as percentage reduction.

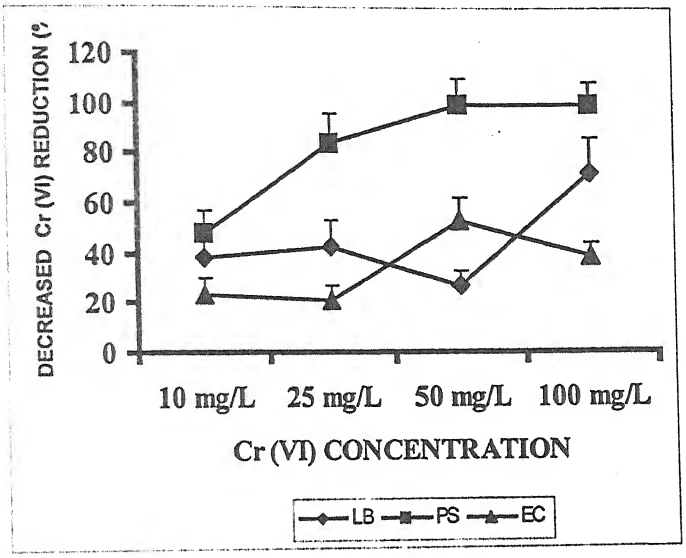


Figure IV.5.4A: Decrease of the capacity to reduce Cr (VI) at the 6th hour by the bacteria obtained from the Cr-stressed rats. The data from Figure 3 was analyzed to get the extent of decrease in the capacity to reduce Cr (VI) in Cr-stressed bacteria as compared to Cr (VI) reduction by bacteria from normal rats. The data has been presented as mean value ±SD of the % decrease.

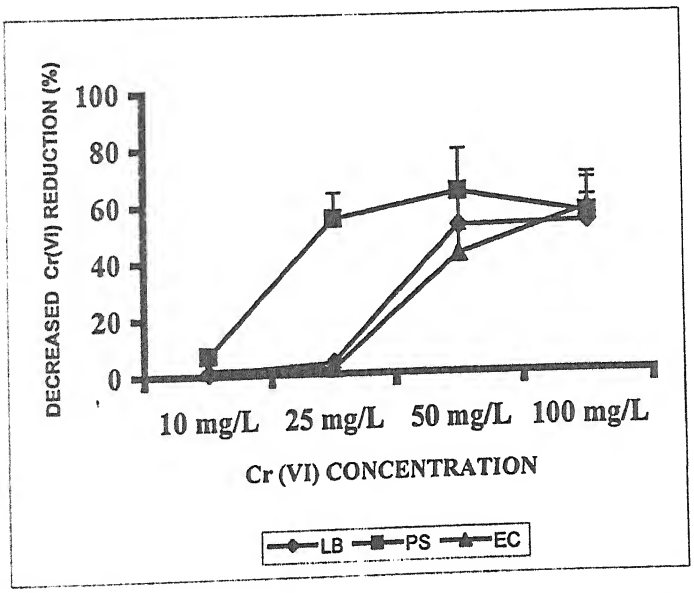


Figure IV.5.4B: Decrease of the capacity to reduce Cr (VI) at the 24th hour by the bacteria obtained from the Cr-stressed rats as in Figure IV.5.4A

Minimum inhibitory concentration (MIC) of Cr (VI): The Findings summarized in Table IV.5.1 show the MIC values of the different bacteria. The least tolerant to the presence of Cr (VI) in the media were the *Lactobacilli*. The MIC Values for *Pseudomonas* sp. obtained from the normal and Cr-stressed rats were 100±20 and 145±28 mg/L, respectively and were the highest. *Escherichia coli* and *Lactobacilli* had more or less similar MIC values. Curing with acridine orange abrogated tolerance to Cr in all the three bacteria (Table IV.5.1).

Table IV.5.1: Minimum inhibitory concentration of chromium

@Source of Bacteria	*Chromium (VI) Concentration mg/L		
	Lactobacillus sp.	Pseudomonas sp.	*Escherichia coli*
Normal	70±25	80±20	65±18
Cr-stressed	120±12	145±28	126±32
#Cured	50±35	60±32	53±28

***Mean values ± SD of the minimum concentration of Cr (VI) that inhibited the growth of the bacteria.**

@The resident bacteria obtained from the gut of normal and Cr-stressed rats.

One group of rat was fed water containing 10 ppm of Cr (VI) (Cr-stressed) and the second group received plain water. The rats were sacrificed after 10 weeks with aseptic precautions and the caecal bacteria were isolated.

#The bacteria were cured with acridine orange

Effect of Cr-stress on antibiotic sensitivity: The antibiotic sensitivity pattern of the three bacteria is presented in Table IV.5.2. It was observed that the Cr-stressed Lactobacilli developed resistance to chloramphenicol, streptomycin and Nalidixic acid while the Cr-stressed Pseudomonas developed resistance to Tetracycline and Kanamycin. The *E. coli* was least affected and showed development of resistance to Kanamycin only. When the bacteria were cured with acridine orange, resistance to antibiotics was abrogated in all the three bacteria.

Table IV.5.2: Comparison of the antibiotic sensitivity pattern of the resident bacteria obtained from the gut of normal and Cr-stressed rats

Antibiotics (ug/ml)	*Lactobacillus sp.			*Pseudomonas sp.			*Escherichia coli		
	Normal	Cr-stressed	#Cured	Normal	Cr-stressed	#Cured	Normal	Cr-stressed	#Cured
Tetracycline (30)	S	S	S	S	R	S	S	S	S
Cotrimazol (25)	S	S	S	R	R	S	R	R	S
Trimethophorin (5)	S	S	S	R	R	S	R	R	S
Chloremphenicol (30)	S	R	S	S	S	S	S	S	S
Streptomycin (30)	S	R	S	S	S	S	S	S	S
Gentamicin (10)	S	S	S	S	S	S	S	S	S
Nalidixic acid (45)	S	R	S	S	S	S	R	R	S
Ampicillin (50)	R	R	S	R	R	R	R	R	S
Kanamycin (30)	S	S	S	S	R	S	S	R	S

***The resident bacteria obtained from the gut of normal and Cr-stressed rats. One group of rat was fed water containing 10 ppm of Cr (VI) (Cr-stressed) and the second group received plain water. The rats were sacrificed after 10 weeks with aseptic precautions and the caecal bacteria were isolated.**

#The bacteria were cured with acridine orange

DISCUSSION

The most significant finding of the present study was the stimulation of growth of facultative gut bacteria from the Cr-stressed rats, and also the significant increase of growth even in presence of lower concentrations of Cr. Furthermore, the capacity to reduce Cr (VI) was significantly decreased along with the increased tolerance of the bacteria to Cr (higher MIC values), which was associated with the development of antibiotic resistance. It has been reported that human ingestion of Cr (VI) in drinking water at levels of 1 to 10 ppm is safe due to high capacity of gastro-intestinal tract to reduce Cr (VI) to Cr (III) (Mirsalis *et al.*, 1996; Shrivastava *et al.*, 2003). In long-term studies, rats are not adversely affected by 2.4 mg/kg/day of Cr (VI) as potassium dichromate in drinking water (Ivankovic and Preussmann, 1975). The exposure dose of 10 ppm used in the present study was non-toxic and more naturalistic.

A number of mechanisms have been reported by which Cr (VI) is reduced to Cr (III). *In vitro* and under physiologic conditions, ascorbic acid, thiols, glutathione, cysteine, cysteamine, lipoic acid coenzyme A, and coenzyme M reduce Cr (VI) at a significant rate (Hamilton and Wetterhahn, 1988). The *in vitro* reaction of Cr (VI) with glutathione results in the formation of a Cr (V) intermediate that is possibly the form that interacts with cellular macromolecules (Jennette, 1982). DT-diaphorase is a major cytosolic enzyme involved in Cr (VI) reduction (DeFlora *et al.*, 1985). The NADPH-dependent Cr (VI) reductase activity of rat liver microsomes has been attributed to cytochrome P-450, whereas the Cr (VI) reductase activity of rat liver mitochondria is attributed to NADH-ubiquinone oxidoreductase (complex I) (Hamilton and Wetterhahn, 1988). Suzuki *et al.*, (1992) showed that the Cr (VI) reductase reduced Cr (VI) to Cr (III) with at least two reaction steps via Cr (V) as an intermediate. The mechanisms of Cr (VI) reduction in the bacteria of the present study need further exploration.

It was observed that the capacity to reduce Cr (VI) was declined in all the three bacteria obtained from the Cr-stressed rats and the loss of the capacity to reduce Cr (VI) was maximum in *Pseudomonas* but had comparatively little effect on *E. coli*. With the increasing concentrations of Cr (VI) the time taken extended and the extent of Cr (VI) reduced was declined. The data revealed that the curves shifted to the right and were reduced in height with the bacteria obtained from Cr-stressed rats. Further analysis showed that the *Pseudomonas* regained the capacity to reduce Cr (VI) with increasing period of incubation. Widespread bacterial reduction of Cr (VI) to the less toxic Cr (III) ions is well known (Horitsu *et al.*, 1987; Cervantes and Silver, 1992; Dhakephalkar *et al.*, 1996; Francisco *et al.*, 2002). In different bacteria, chromate reduction is either an aerobic or an anaerobic process (but not both) and is carried out either by soluble proteins or by cell membranes (Cervantes and Silver, 1992).

The growth profile of the *Pseudomonas* and *Lactobacillus* obtained from the Cr-stressed rats had significantly increased growth in comparison with that of the bacteria from the normal rats. On the other hand, *E. coli* showed comparatively little difference in the growth rate of the bacteria obtained from the normal control rats and the Cr-stressed rats. A significant reduction in doubling time with a significant increase in number of generations and increase in specific growth rate of Cr-stressed *Pseudomonas* and *Lactobacillus* throughout the growth period as compared to their respective bacteria from the normal rats was also observed (data not shown). Similar results were obtained when the two groups of the bacteria were grown in presence of lower Cr concentrations. However, these changes were not observed in case of Cr-stressed *E. coli*. The results of doubling time and number of generations of Cr-stressed and bacteria from normal animals indicate bacterial specificity with respect to Cr-stress. The interesting finding of the stimulated growth of Cr-stressed *Pseudomonas* and *Lactobacillus* and complete Cr (VI) reduction capacity (up to 25 mg/L Cr-concentration) indicate their ability to adaptation.

This may also reflect horizontal genetic transfer resulting from Cr-stress. Diversity of Cr-resistant and Cr-reducing bacteria in the bacterial population from a chromium contaminated activated sludge has been established (Francisco *et al.*, 2002). It has also been suggested that the mechanisms of Cr (VI) resistance and reduction may differ in microbial community from group to group. Therefore, Cr (VI) resistance and reduction capacity could be the shared abilities and not an exclusive characteristic of a single group of bacteria. Present findings showing the development of multiple resistances in *Lactobacillus* and *Pseudomonas* obtained from Cr-stressed rats to various antibiotics as compared to *E. coli*, which showed resistance to Kenamycin only, further indicates the specificity of resident gut microflora under Cr-stress.

In the cells of higher animals intracellular Cr (VI) is metabolically reduced to the ultimate Cr (III) resulting in the formation of reactive intermediates that contribute to the cytotoxicity, genotoxicity and carcinogenicity. A series of *in vitro* and *in vivo* studies have demonstrated that a cascade of cellular events occur following Cr (VI)-induced oxidative stress including enhanced production of super oxide anion and hydroxyl radicals, increased lipid peroxidation and genomic DNA fragmentation, modulation of intracellular oxidized states, activation of protein kinase C, apoptotic cell death and altered gene expression (Bagchi *et al.*, 2001; Shrivastava *et al.*, 2002) The ability of microorganisms to alter their chemical physiology in order to compensate for potentially traumatic changes in their external environment represents a built in factor of safety for biological survival. Growth of *E. coli*, *Micrococcus luteus* and *Azotobactor* sp. in the presence of lead and growth of *Chlamydomonas reinhardi* in the presence of mercury are examples of biological accommodation (Ben-Bassat *et al.*, 1972; Tornabene and Edwards, 1972; Kumar and Upreti, 2000). Growth stimulation of facultative *Methylobacterium* sp. following the addition of molybdenum or tungsten, which enhances the intracellular formate dehydrogenase activity, has also been

reported (Girio *et al.*, 1998). Enzymatic reduction of Cr (VI) by hexavalent Cr-tolerant *Pseudomonas ambigua* G-1 isolated from activated sludge has been reported. The intracellular reducing enzyme required NADH as a hydrogen donor (Horitsu *et al.*, 1987). A membrane associated chromate reductase activity from *Enterobacter cloacae* isolated from activated sludge has also been documented (Wang *et al.* 1990). In the present context of Cr-interaction with facultative gut bacteria further studies are required to identify cellular and molecular mechanisms of accommodation / adaptation as well as stimulation of growth of Cr-stressed *Pseudomonas* and *Lactobacillus* sp. Cr (VI) is highly toxic to all forms of living organisms and is mutagenic in bacteria (Losi *et al.*, 1994). Some bacteria are known to bioaccumulate up to 34 mg Cr/g dry weight Srinath *et al.*, 2002). Intracellular accumulation of such large amount of Cr may disturb the normal functioning of the bacterial cell. On the other hand chromium is an essential micronutrient required to promote the action of insulin in body tissues so that the body can use sugars, proteins and fats. Cr (III) salts such as chromium polynicotinate, chromium chloride and chromium picolinate, are used as micronutrients and nutritional supplements, and have been demonstrated to exhibit a significant number of health benefits in animals and humans Guthrie, 1982; Anderson, 2000). Several factors including mutagenic potential of Cr, a Cr (VI) reductional intermediate product for example Cr (III), a reaction to accumulated Cr or a selective stimulation of the control of binary fission, membrane proteins and lipids interaction and other intracellular mechanisms may play important role.

The findings further show that *Pseudomonas* obtained from the Cr-stressed rat had the highest MIC value while the *Lactobacillus* and *E. coli* had lower values. As compared to the bacteria from the normal control rats the MIC values were significantly higher in the Cr-stressed rats. This indicated that bacteria from Cr-stressed animals tolerated the presence of Cr in the milieu much better. In a recent study 16% of the Cr (VI) resistant bacterial strains

isolated from tannery effluent had MIC more than 100 mg Cr (VI)/L (Srinath *et al.*, 2002). Another study reports that under *in vitro* conditions, Cr-tolerance may depend on the type of media used, the MIC obtained in the rich media are from two to five times higher than in minimal media because heavy metals can be complexed by some components of media, specially organic substances and phosphate (Mergeay, 1995). Bacterial sensitivity to metal toxicity is known to depend on their isolation site. In natural bacterial communities, the development of metal resistance is greatly enhanced by the horizontal dispersal of genetic information (Schmidt and Schlegel, 1989). Evolution of resistance via such transfer between natural bacterial isolates has been shown to occur *in situ* and also under laboratory conditions (Top *et al.*, 1990).

A number of components in the intestines may be responsible for efficient handling of Cr (VI). We have also shown that Cr (VI) is efficiently reduced under *in situ* intestinal incubation and also by upper villus and middle villus cells of rat intestine. The present study indicates that the gut bacteria have marked capacity to cope with the increased load of chromium and may contribute in the protection against chromium toxicity up to certain extent. In addition, various antibiotic resistance shown by resident gut bacteria following Cr-ingestion also indicates that use of chromium as nutritional supplement / micronutrient may provide significant protection to the gut microflora, particularly *Lactobacillus*, against some of the commonly used antibiotics. The antibiotic resistance developed in the *Lactobacillus* may be a blessing in disguise as the bacteria may continue to provide benefits even in patients given antibiotic therapy. The gut is the natural habitat for a large and dynamic bacterial community. Major functions of the gut microflora include metabolic activities that result in salvage of energy and absorbable nutrients, important trophic effects on intestinal epithelia and on immune structure and function, and protection of the colonized host against invasion by alien microbes (Guarner and Malgelada, 2003). A prebiotic substance has been defined as a

non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon. Therefore, compared to probiotics, which introduce exogenous bacteria into the colonic microflora, a prebiotic aims at stimulating the growth of one or a limited number of the potentially health-promoting indigenous micro-organisms, thus modulating the composition of the natural ecosystem (reviewed by Cashman, 2003). The finding of the present study indicates that chromium may act as a prebiotic. In recent years, increasing attention has been focussed on the possible beneficial effects of prebiotics.

However, altered functions of resident gut microflora following chronic exposure of chromium cannot be ruled out. This, in turn, may adversely affect the body by depriving it of the benefits provided by the microflora that may manifest clinically as various nutritional deficiency syndromes. Thus resident gut microflora plays a very important role in protection against Cr toxicity.

Conclusion

Summary and Conclusion

SUMMARY AND CONCLUSIONS

Dengue virus (DV) infection is endemic all over India so is the occupational and non-occupational exposure to hexavalent chromium Cr(VI). The pathogenesis of dengue depends upon the immune response of the body. The intermediary products generated during reduction of Cr(VI) kill the target cells including leucocytes by apoptosis. This effect of chromium compromises the immune response of the host. It is, therefore, possible that the chromium toxicity may affect the disease process during dengue virus infection. There are no reports in literature on the outcome of dengue virus infections during chromium toxicity, therefore, the present study was undertaken. An attempt has been made to answer the following questions: What are the effects of subtoxic dose of Cr (VI) on the peripheral blood cells of mice during dengue virus infection? Does Cr (VI) toxicity enhance the ill effects of DV on splenic macrophage and lymphocyte functions? Can the cells of immune system detoxify Cr (VI)? Since the commonest mode of entry of Cr (VI) in the body is through ingestion, the question answered was can the intestinal cells and the gut microflora help in detoxifying the ingested Cr(VI). These questions have been answered using Swiss mice and Wistar rats that were bred and maintained in the animal facility of the Institute. Mice were used for DV plus Cr (VI) studies as it is the animal of choice for the viral studies while rats have been used extensively for Cr (VI) studies.

Chromium enters the body through the lungs, gastro-intestinal tract, and to a lower extent through skin and most of the chromium absorbed is distributed in the lungs, liver, kidneys, RBC, plasma, spleen, bone-marrow. The main routes for the excretion of chromium are via kidney/urine and the bile/feces. The presence of chromium in different organs of body affects their functions the commonest to be affected are the peripheral blood cells. During dengue virus infection also various changes occur in the peripheral blood cells.

These experiments were therefore, conducted to study the effect of chromium (VI) on peripheral blood cells during dengue virus infection of mice. Mice maintained on pellet diet in the animal house of this Institute were used. One group of mice were given *ad-lib* drinking water containing 10 ppm of Cr (VI) and the second group was given plain water to drink. At the 3, 6 and 9 week of Cr (VI) drinking, a set of mice from each group were inoculated ic with DV in doses of 1000 LD_{50}. The mice were killed in groups of 6 at the 4^{th} and 8^{th} day of the virus inoculation and various investigations were done.

The most significant findings of these experiments are reduction in lymphocyte percentage and increase in the granulocyte, monocyte and platelet counts in mice fed Cr(VI) with drinking water. Differential leucocyte counts revealed that percentage of lymphocytes and the absolute lymphocyte numbers were decreased by 50%. The percentage of polymorphonuclears and monocytes was increased gradually from 3 to 9 weeks. The increase in absolute monocyte number was four folds while that of polymorphonuclears was two folds at the 9^{th} week of Cr(VI) drinking.

The normal mice inoculated i.c. with 1000 LD_{50} of DV showed a 48% decrease in total leucocyte count which was mainly due to the reduction in the absolute numbers of lymphocytes. When Cr(VI) fed mice were inoculated with DV, total leucocyte count was decreased more or less similar to the normal mice inoculated with DV. The interesting finding was marked reduction in the absolute lymphocytes count while the effect on the number of polymorphonuclear cells was insignificant.

By drinking Cr(VI) for 3 weeks the red blood cell count was increased by 29% while at 6^{th} and 9^{th} weeks it remains similar. A marked reduction in mean corpuscular volume (18%) and mean corpuscular haemoglobin (15%) was observed at the end of 3^{rd} week of Cr(VI) drinking. After 9 weeks of drinking Cr(VI) haematocrit (32%) and haemoglobin (23%) contents were decreased. The mechanism of decrease of haemoglobin appears to be due to

inhibition of its biosynthesis by Cr (VI) by the DNA damage, producing inhibition of the activities of one or more enzymes involved in heme synthesis. Haemoglobin concentration was decreased by 42% at the 8th day in normal mice inoculated with DV. The haematocrit value was also decreased by 40%. Gastrointestinal bleeding may initially be occult and usually manifest as a drop in haematocrit without clinical improvement.

When Cr(VI) was given to mice the platelet counts were increased gradually from 3 to 9 weeks, the increase being 90% at the 9th week. When normal mice were inoculated with DV, platelet counts were decreased by 50% and 40% at the 4th and 8th day post inoculation respectively. The cause of thrombocytopenia is either impaired megakaryocytes production or increased platelet destruction. When Cr(VI) treated mice were inoculated with DV the platelet count decreased but the decrease was lesser than the normal mice given DV. This showed that drinking of Cr(VI) prevented the fall in platelet count during DV infection.

Spleen is one of the larger lympho-reticular organs that plays important role in the defence mechanisms of body. In this series of experiments the effects of Cr (VI) and DV were studied on the spleen and its cell in mice. The findings of this study showed a significantly reduction in the weight of spleen following oral exposure of mice to subtoxic doses of Cr(VI). The reduction in the weight of spleen was gradual with the increasing period of Cr(VI) feeding and was maximum at the 9th week, the reduction being 51%. A significant reduction (32 to 34%) in the weight of spleen was observed when normal control mice were inoculated with DV. On the other hand when Cr (VI) fed mice were inoculated with DV the weight of spleen was increased as compared to that in mice fed Cr(VI) alone or inoculated with DV alone. The reason of this apparent increase in spleen weight appears to be due to the greater decrease in the body weight of mice.

During dengue virus infection the $CD4^+$ T lymphocytes produce a unique cytokine, Cytotoxic Factor (CF). CF appears to be pathogenesis-related proteins, capable of reproducing DHF-like pathological lesions in mice, such as increased capillary permeability, cerebral edema, and blood leukocyte changes. CF is present in the homogenates prepared from the DV-infected mouse spleen and has the capacity to kill normal mouse spleen cells in one-hour time. The experiments done to detect the presence of CF show that the cytotoxic activity of spleen homogenate of DV-infected mice was up to 23%. The cytotoxic activity of spleen homogenates obtained from mice fed Cr(VI) for 3 weeks was 33±6% and that of Cr(VI) plus DV were 54%. Such summation of cytotoxic effect was not seen at 6 and 9 weeks as the cytotoxic activity of Cr(VI) fed mice was very high. Cr(VI) is carcinogenic and mutagenic. Toxic effects of Cr (VI) in *in vivo* and *in vitro* are related to its intracellular fate. A series of *in vitro* and *in* vivo studies have demonstrated that Cr(VI) induces an oxidative stress through enhanced production of reactive oxygen species (ROS) leading to genomic DNA damage and oxidative deterioration of lipids and proteins and apoptotic cell death.

The phagocytic activity of the splenic macrophages in the different groups of mice was studied using Latex beads. In normal control mice phagocytic activity of the splenic macrophages was 92±3%. When normal mice were inoculated DV, the phagocytic activity reduced to 65±6 and 45±8% at the 4^{th} and 8^{th} day post inoculation. On the other hand greater reduction in phagocytic activity was also seen in the splenic macrophages of Cr(VI) treated mice, the maximum being 36±7% at the 9^{th} week. A comparison of the phagocytic activity of the splenic macrophages of mice drinking Cr(VI) water and inoculated with DV showed greater reduction of at that the 9^{th} week..

In another series of experiments the mitogenic stimulation of the spleen cells by Concanvalin A in the different groups of mice was studied. Stimulation was presented as thymidine uptate by spleen cells in counts per minute. When

normal mice were inoculated with DV maximum proliferation was occurred at the 4^{th} day but were reduced to the level of normal controls by the 8^{th} day. On the other hand after 9 weeks of drinking Cr(VI) spleen cell proliferation was increased by two folds of normal mice spleen cells. When Cr(VI) treated mice were inoculated with DV proliferation observed less than Cr group but more than DV inoculated group.

The findings of these experiments show that both, DV and Cr (VI) have damaging effects on the cells of peripheral blood and the spleen except the platelets. Presence of Cr (VI) in body reduces the damaging effects of DV on the platelets.

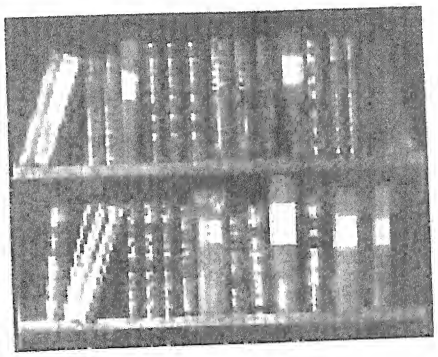
The findings of the above experiments led to question of the role of various cells of body in handling the Cr (VI). The cells of the immune system form a strong line of defence against foreign substances. A study was undertaken to investigate the capacity of different cells of Wistar rats to reduce potentially carcinogenic hexavalent chromium (Cr-VI) into less toxic trivalent chromium *in vitro*. $5x10^6$ cells were incubated with 10 or 25 µg/ ml of Cr (VI) in the form of $K_2Cr_2O_7$ at 37°C in presence of 5% CO_2 in air. At various time periods the remaining amount of Cr (VI) was measured and the percentage of Cr (VI) reduced was calculated. Among the single cell suspensions from the splenic cells a peak reduction of 55% was observed with the total spleen cells, 40% with the B-lymphocyte-enriched subpopulation, 10% with T-lymphocytes and 24% with the macrophages. The reduction by splenic and peritoneal macrophages was similar. Total thymocytes reduced 54% of the Cr (VI). The time taken by each cell type for the peak reduction to Cr (VI) was markedly different. The finding thus show, that the capacity of different cells in the body differ vastly in their capacity and time taken to reduce hexavalent chromium.

Since the most common route of entry of chromium is through drinking water and food, intestinal cells were also investigated. Another series of experiments were undertaken to investigate the capacity of different cells of the intestines of Wistar rat to reduce Cr-VI into less toxic trivalent chromium *in vitro*. $5x10^6$ cells were incubated with 10 or 25 µg/ ml of Cr (VI) in the form of $K_2Cr_2O_7$ at 37°C in presence of 5% CO_2 in air. At various time periods the remaining amount of Cr (VI) was measured and the percentage of Cr (VI) reduced was calculated. Among the intestinal cells the maximum reduction of 100% (of 10 µg/ml) was observed with the upper villus cells and 72% with the middle villus cells while reduction was the least (4%) with the Crypt cells. The reduction in the intestinal loop *in situ* was 100%. The time taken by each cell type for the peak reduction to Cr (VI) was markedly different. The finding thus show, that the capacity of different cells in the body differ vastly in their capacity and time taken to reduce hexavalent chromium. The most efficient handling of Cr (VI) by the intestine, due to the presence of a variety of cells, protects the body from its adverse effects even after chronic exposure.

The major non-occupational source of chromium (Cr) for humans is through ingestion with food and water but its effect on the gut microflora has not been studied. The present experiments were, therefore, undertaken to investigate the effects of chronic ingestion of Cr(VI) on the resident gut microflora of Wistar rats. A group of rats were kept on drinking water containing 10 ppm chromium VI (Cr (VI)) (called Cr-stressed animals) and the other group was given plain water. After 10 weeks *Lactobacillus, Pseudomonas* sp. and *Escherichia coli* were isolated from the caecum of the rats and various studies were performed. The most significant findings of the present study were the stimulation of growth of facultative gut bacteria from the Cr-stressed rats, and also the significant increase of growth even in presence of lower concentrations of Cr. Furthermore, the capacity to reduce Cr (VI) was significantly decreased along with the increased tolerance of the

bacteria to Cr (higher minimum inhibitory concentration (MIC) values), which was associated with the development of antibiotic resistance. The effects were most marked with the *Pseudomonas* sp. and least with the *Escherichia coli.* The antibiotic resistance developed in the *Lactobacillus* may be a blessing in disguise as the bacteria may continue to provide benefits even in patients given antibiotic therapy. It appears that the changes were a sequel to the effort of gut bacteria to provide the first line of defense to the body by converting toxic Cr (VI) to a less toxic Cr (III) and may act as prebiotic.

The findings of the present study, thus show that body makes an effort to detoxify Cr(VI) by various cells and the gut microflora. Inspite of this, presence of even subtoxic doses of Cr (VI) in the body can enhance the severity of a virus infection.



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Appendix

APPENDIX

Method for measurement of Cr (VI)

0.5 ml of sample +250 µl. of 5N H_2SO_4 + 1.85 ml. distilled water in a tube, vortexed properly and then added 400 µl. diphenyl carbazide (freshly prepared) as coloring reagent

Took OD at 540 nm.

Diphenyl carbazide

12.5 mg of diphenyl carbazide (Sigma) was dissolved in 5 ml. of acetone. This solution was prepared just before the experiment and protected from light.

Preparation of stock solution of Con A

1 mg of Concanavalin purchased from CalBiochem was dissolved in 1 ml. of MEM. Stored in small aliquots at –20°C.

Preparation of MTT stock solution

MTT (3-(4-5 dimethylthizol-2-yl)-2-5 diphenyl tetrazolium bromide, Sigma catalog no. M2128) was dissolved in PBS at 5 mg/ml. and filtered to sterilize and remove a small amount of insoluble residue present in solution. Stock was stored at 4°C in small aliquots.

Trypan blue dye

Prepared 10% stock solution of trypan blue in sterilized PBS (pH 7.2)

Scintillation liquid (for 650 ml.)

Methanol- 150ml.

Toluene- 250ml.

Dioxane- 250 ml.

Naphthalene- 25 mg.

PPO - 3.25 mg.

POPOP - 0.065 mg.

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PPO - 3.25 mg.

POPOP- 0.065 mg.

All solvents and chemicals were of scintillation grade and purchased from Qualigens.

Phosphate Buffered Saline

NaCl	8 gm/lit
K_2HPO_4	1.2 gm/lit
KH_2PO_4	0.34 gm/lit

Adjusted the pH 7.2 with 0.1N NaOH and 0.1 N HCl

LIST OF PUBLICATIONS

1. **Shrivastava R,** Upreti RK, Seth PK, Chaturvedi, UC. Effects of chromium on the immune system. FEMS Immunol Med Microbiol, 2002; 34: 1-7.

2. **Shrivastava R,** Upreti RK, Chaturvedi, UC. Various cells of immune system and intestine differ in their capacity to reduce hexavalent chromium. FEMS Immunol Med Microbiol, 2003; 38: 65-70

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MiniReview

Effects of chromium on the immune system

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Abstract

Chromium is a naturally occurring heavy metal found commonly in the environment in trivalent, Cr(III), and hexavalent, Cr(VI), forms. Cr(VI) compounds have been declared as a potent occupational carcinogen among workers in chrome plating, stainless steel, and pigment industries. The reduction of Cr(VI) to Cr(III) results in the formation of reactive intermediates that together with oxidative stress oxidative tissue damage and a cascade of cellular events including modulation of apoptosis regulatory gene p53, contribute to the cytotoxicity, genotoxicity and carcinogenicity of Cr(VI)-containing compounds. On the other hand, chromium is an essential nutrient required to promote the action of insulin in body tissues so that the body can use sugars, proteins and fats. Chromium is of significant importance in altering the immune response by immunostimulatory or immunosuppressive processes as shown by its effects on T and B lymphocytes, macrophages, cytokine production and the immune response that may induce hypersensitivity reactions. This review gives an overview of the effects of chromium on the immune system of the body.

Keywords: Chromium; Immune response; T lymphocyte; B lymphocyte; Macrophage; Apoptosis; Cytotoxicity; Micronutrient

1. Introduction

A large number of biologically active substances, including heavy metals, may have direct, primary or secondary effects on the immune system and are of interest to pathologists, immunologists and toxicologists. Various metals are responsible for many biochemical, immunological and physiological activities of the body as micronutrients. But some of them can give rise to disordered functions of the immune system resulting in increased susceptibility to infection, a variety of hypersensitivity reactions, autoimmune diseases and neoplasia. Heavy metals are of significant importance in altering the immune response by immunostimulatory or immunosuppressive mechanisms.

Chromium is a naturally occurring heavy metal found in the environment commonly in trivalent, Cr(III), and hexavalent, Cr(VI), forms. The reduction of Cr(VI) to Cr(III) results in the formation of reactive intermediates that contribute to the cytotoxicity, genotoxicity and carcinogenicity of Cr(VI)-containing compounds. The major non-occupational source of chromium for humans is food such as vegetables, meat, urban air, hip or knee prostheses and cigarettes [1,2]. Cr(VI) is a widely used in industrial chemicals, extensively used in paints, metal finishes, steel including stainless steel manufacturing, alloy cast irons, chrome and wood treatment. On the contrary, Cr(III) salts such as chromium polynicotinate, chromium chloride and chromium picolinate (CrP) are used as micronutrients and nutritional supplements and have been demonstrated to exhibit a significant number of health benefits in animals and humans [3].

Chromium enters the body through the lungs, gastrointestinal tract and to a lesser extent through skin. Inhalation is the most important route for occupational exposure, whereas non-occupational exposure occurs via ingestion of chromium-containing food and water. Regardless of route of exposure Cr(III) is poorly absorbed whereas Cr(VI) is more readily absorbed. Further, absorption of Cr(VI) is poorer by oral route, it is thus not very toxic when introduced by the oral route. But chromium is very toxic by dermal and inhalation routes and causes lung cancer, nasal irritation, nasal ulcer, hypersensitivity reactions and contact dermatitis. All the ingested Cr(VI) is reduced to Cr(III) before entering in the blood stream. The main routes for the excretion of chromium are via kidney/urine and the bile/feces [1,2,4]. Cr(III) is unable

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Suboptimal chlorine treatment of drinking water leads to selection of multidrug-resistant *Pseudomonas aeruginosa*

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Abstract

The present study was undertaken to investigate the spectrum of bacteria present in the River Gomti water before and after chlorination for drinking purposes. We observed that the strains of *Pseudomonas aeruginosa* that survived chlorination on three out of seven occasions were resistant to almost all the antibiotics tested. The chlorine-resistant bacteria had mucoid colonies and grew better at 24°C. All attempts to isolate the plasmid responsible for chlorine resistance were unsuccessful. Laboratory experiments using different strains of the *P. aeruginosa* in distilled water showed that only the resistant strain survived chlorine treatment at a dose of $\leqslant$500 μg/L. Similar results were obtained when water collected from seven different sites on the River Gomti was treated with graded doses of chlorine. At the higher dose of chlorine, all the bacteria died in 30 min, whereas with lower doses all the bacteria survived. The present study underscores the importance of measuring water chlorine concentrations to assure they are sufficiently high to remove pathogenic bacteria from drinking water. To our knowledge, this is the first report in the literature of the selection of multidrug-resistant bacteria by suboptimal chlorine treatment of water.

Keywords: River water; *Pseudomonas aeruginosa*; Chlorine treatment; Multidrug resistance

1. Introduction

Chlorine is added to drinking water to reduce or eliminate microorganisms, which can be present in water supplies. Most municipal water supplies are chlorinated with chlorine gas. Swimming pools, hot tubs, and the like are usually chlorinated with chlorine-containing substances such as calcium hypochlorite, sodium hypochlorite (bleach), or trichloro-*S*-triazinetrione (commonly known as "trichlor"). In every case, the effectiveness of chlorine as a germicide is a result of chlorine's powerful oxidizing action. The addition of chlorine to drinking water has greatly reduced the risk of waterborne diseases. For more than a century, the safety of drinking water supplies has been greatly improved by chlorine treatment. Still, chlorine remains the most commonly used drinking water disinfectant.

A number of bacteria have been shown to develop resistance to different agents used for the treatment of water, including chlorination (Ridgway and Olson, 1982; Pyle et al., 1994; Le Dantec et al., 2002) and sodium dichloroisocyanurate (D'Auria et al., 1989). Maillard et al. (1998) have reported resistance of *Pseudomonas aeruginosa* PAO1 phage F116 to sodium hypochlorite. Ridgway and Olson (1982) reported that the most sensitive bacteria including *Pseudomonas* spp. are readily killed by chlorine concentrations of $\leqslant$1.0 mg/L. Pyle et al. (1994) have isolated a *P. cepacia* strain that has reduced susceptibility to iodine and to chlorination. Stewart et al. (2001) have reported that bacteria in the biofilms of *P. aeruginosa* and *Klebsiella pneumoniae* are highly resistant to killing by both alkaline hypochlorite and chlorosulfamates.

P. aeruginosa is an opportunistic pathogen that is known to cause urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, and a variety of systemic infections, particularly in patients with severe burns, and in cancer

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P. aeruginosa is an opportunistic pathogen that is known to cause urinary tract infections, respiratory system infections, dermatitis, soft tissue infections, bacteremia, and a variety of systemic infections, particularly in patients with severe burns, and in cancer

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Review Article

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Gut microflora & toxic metals: Chromium as a model

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The gastrointestinal tract (GIT) is exposed to various environmental pollutants including metals, that contaminate food and water which may have toxic effects on body. GIT has large amount of microbes that live in symbiosis and help the host in different ways. The resident gut microflora have a significant role to play in detoxification and elimination of the harmful metals from the body. Chromium is a naturally occurring heavy metal found commonly in environment in trivalent (Cr III) and hexavalent (Cr VI) forms. Cr (VI) compounds have been shown to be potent occupational carcinogens. The reduction of Cr (VI) to Cr (III) results in the formation of reactive intermediates that together with oxidative stress and oxidative tissue damage, and a cascade of cellular events including modulation of apoptosis regulatory gene p53 contribute to the cytotoxicity, genotoxicity and carcinogenicity of Cr(VI)-containing compounds. The data discussed here with reference to chromium show that gut microflora have a marked capacity to cope with the increase load of ingested metals and may contribute significantly in the protection against metal toxicity.

Key words Chromium (VI) reduction - chromium resistance - *Escherichia coli* - gut microflora - intestinal bacteria - lactobacillus prebiotic - probiotic - toxic metals

The gastro intestinal tract (GIT) is exposed to different environmental pollutants that contaminate food and water. These include metals that may have toxic effects on body. Many metals have no known biological function and some of these the capable of disrupting essential physiological processes. Examples include arsenic, cadmium, lead, chromium and mercury. Some metals also serve a chemically important role as essential components of many enzymes. These metalloenzymes are involved in the synthesis, repair and degradation of biological molecules, release and recognition of certain biological signaling molecules, and transfer of small molecules and electrons in crucial processes such as in respiration. For example, iron-containing haemoglobin transports oxygen in blood. The toxic effects of most metals can be traced due to their ability to disrupt the function of essential biological molecules, such as proteins, enzymes and DNA. In some cases this involves displacing chemically related metal ions that are required for important biological functions such as cell growth, division and repair.

Certain heavy metals form very stable and long-lasting complexes with sulphur in biological molecules, which can disrupt their biological function. In some cases these metals may be concentrated at higher levels of the food chain. The body has developed various mechanisms to detoxify the toxic substances, including the metals and the cells and the secretions of GIT play an important role in this process. The cells have evolved a complex network of metal trafficking pathways. The object of such pathways is to prevent accumulation of the metal in the freely reactive form (metal detoxification pathways) and to ensure proper delivery of the ion to target metalloproteins (metal utilization pathways)[1]. In recent times, microbes have been shown to reduce a wide range of toxic metals *viz.*, chromium [Cr(VI)], mercury [Hg(II)], cobalt [Co(III)], lead [Pb(II)], and

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Guest Editorial

DENGUE HAEMORRHAGIC FEVER: A GLOBAL CHALLENGE

*UC Chaturvedi, R Shrivastava

Dengue haemorrhagic fever (DHF) is back once again. Prior to 1970 only nine countries had experienced DHF epidemics. The prevalence of dengue has grown dramatically in recent decades. The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia and the Western Pacific. Southeast Asia and the Western Pacific are most seriously affected. Some 2500 million people - two fifths of the world's population - are now at risk from dengue. WHO currently estimated 50 million cases of dengue infection worldwide every year. During epidemics of dengue, attack rates among susceptibles are 40 to 90%. An estimated 500,000 cases of DHF require hospitalization each year, of whom a very large proportion are children.[1,2] The year 2001 witnessed unprecedented global dengue epidemic activity in the American hemisphere, the Pacific islands and continental Asia. During 2002, more than 30 Latin American countries reported over 1,000,000 dengue fever cases with large number of DHF cases. This has been followed by extensive epidemics of DHF in several parts of India during 2003.

Dengue is a mosquito borne virus infection which is found in tropical and subtropical regions around the world, predominantly in urban and semiurban and now in rural areas. Dengue is caused by four distinct viruses (type 1 to 4) that are closely related antigenically. Humans are the main amplifying host of the virus. Recovery from infection provides lifelong immunity against that serotype but confers only partial and transient protection against subsequent infection by the other three. It has been suggested that sequential infection increases the risk of more serious disease resulting in DHF.

The Indian encounter with dengue and DHF is interesting and intriguing. The first major epidemic illness compatible clinically with dengue occurred in Madras in 1780 which later spread to all over the country. The dengue virus was first isolated in Japan in 1943 but the one isolated at Calcutta in 1944 from the blood of US soldiers was considered as a first report for a long time. DHF, a potentially lethal complication of dengue virus infection, was first recognized in the 1950s during the dengue epidemics in the Philippines and Thailand and quickly spread to other parts of the World.[3]

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Today DHF affects most Asian countries and has become a leading cause of hospitalization and death among children in several of them. The risk factors for DHF are infestation with *Aedes* mosquito, hot and humid climate enhancing mosquito breeding, mosquito density, and presence of all the four serotype of the dengue virus with secondary infection in the host, the water storage pattern in the houses, population density and large movement of people towards urban areas. While DHF was present in the neighbouring countries for a long time it was not known why India was not affected since all the risk factors were present in this country. When the first extensive epidemic of DHF occurred during 1996 in Northern India, there was no clue of its emergence.

The 1996 epidemic in India was mainly due to dengue type 2 virus[4] while the 2003 epidemic appears to be mainly due to dengue type 3 virus. Over the past two decades, dengue virus type 3 (DV-3) has caused unexpected epidemics of DHF in Sri Lanka, East Africa and Latin America. Isolates from these geographically distant epidemics are closely related and belong to DV-3, subtype III, which originated in the Indian subcontinent. The emergence of DHF in Sri Lanka in 1989 correlated with the appearance of a new DV-3, subtype III variant. This variant most likely spread from the Indian subcontinent into Africa in the 1980s and from Africa into Latin America in the mid-1990s. DV-3, subtype III isolates from mild and severe disease outbreaks form genetically distinct groups.[5] Which suggests a role for viral genetics in DHF. There is need to genotype the dengue viruses isolated from the different parts of this country and study their relationship. At the same time human leukocyte antigen alleles correlate with both protection and susceptibility to DHF and dengue shock syndrome. Studies in Haiti suggest that blacks have a gene providing nearly complete protection against severe dengue illness.

Early diagnosis of dengue virus infection is important and can be established with easily available laboratory tests. But what is the value and appropriate use of the tourniquet test in dengue? A negative test does not rule out dengue infection, a positive test should be followed by close surveillance for early signs of DHF. Low platelet counts do not predict clinically significant bleeding in dengue. It follows that platelet or blood transfusions should not be administered based upon platelet count alone. DHF or dengue shock syndrome